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L. KESZTYŰS, I. NÁSZ, K. RAUSS, F. B. STRAUB, L. VÁCZI,
J. WEISZFEILER

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G. IVÁNOVICS

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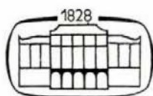
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SYMPOSIUM
ON ORAL ENTERIC BACTERIAL VACCINES

BUDAPEST, APRIL 10—13, 1973

ORAL IMMUNIZATION AGAINST ENTERIC INFECTIONS USING INACTIVATED BACTERIA

A REVIEW

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Summary. A survey is presented of oral immunization with inactivated vaccines against enteric bacterial infections, on the basis of laboratory and field studies.

At this symposium, many specialists from socialist countries have gathered working on problems of oral immunization against intestinal infections using inactivated antigens. To present a survey of this subject before this audience is a most honouring but also quite difficult task. It is not our intention to give a complete summary and critical evaluation of the numerous experimental studies conducted on oral immunization with inactivated bacteria. Such evaluation will be the final result of this symposium. We only wish to comment on the following five questions which seem to us important to be discussed here.

1st question: How long have studies been carried out on this subject and why is it of special interest to-day?

2nd question: What types of vaccines obtained from inactivated bacteria are most commonly used?

3rd question: What criteria are applied to test inactivated bacterial oral vaccines?

4th question: What practical results have been obtained with such vaccines?

5th question: Which problems in the development and application of these vaccines are unsolved?

Question number one: How long have studies been carried out on this subject and why is it of special interest to-day? A study by PAUL EHRLICH in 1891 is considered to be the first scientific work on oral immunization. EHRLICH reported on feeding to mice the vegetable toxins ricin and abrin. By this method he succeeded in producing an increasing resistance to the toxins in the animals. But actually it was METSCHNIKOFF's disciple, ALEXANDER BES-REDKA, who conceived the idea of local immunization.

In his monograph "Local Immunization", which appeared in German in 1926, he put forward the general idea of this immunization procedure: The

antigen is to produce a local immunity at the very site at which the natural infection attacks. BREUEL compiled a list of the pertaining literature from 1891 to 1968 covering 2227 titles. The number of these publications according to the year of their appearance is shown in Fig. 1.

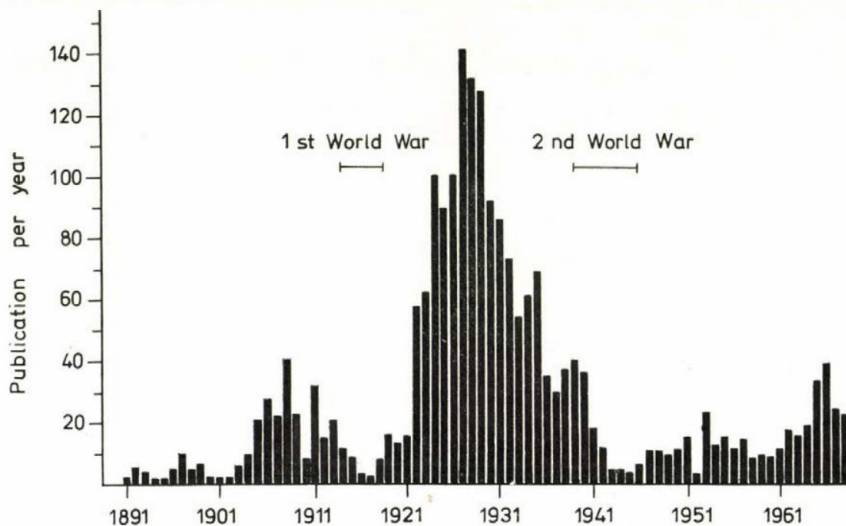


Fig. 1. Increase of the number of publications on local immunization (after BREUEL)

It is seen that already before World War I there were numerous publications about this subject. During the war the number of publications declined. It reached a peak in the subsequent period mainly due to the work of BESREDKA and his school, and decreased again during World War II. This was followed by another rise. In this diagram the most recent voluminous work has not been taken into account. The increasing interest in these questions is reflected also by a number of international symposia, like this one, having been held since 1968, for instance in Wernigerode, Bern and Milan. The 1972 report of a WHO Scientific Group states the following reasons for these increasing scientific activities.

(1) The success of oral immunization against poliomyelitis has served as an important stimulus to try this approach against enteric bacterial infections.

(2) At present there are no completely satisfactory parenteral vaccines against bacterial infections of the intestines.

The efficacy of these vaccines has not been established with certainty. The duration of immunity induced by them is insufficient. They cause side reactions and the cost involved to immunize the whole population is considerable.

(3) Oral immunizing procedures are likely to be less expensive, and these vaccinations can more easily be organized by health authorities. However, there are still some open questions regarding the efficacy of such vaccinations, the duration of protection, and the safety of their performance. And this leads us to the second question. What types of vaccines obtained from inactivated bacteria are most commonly used?

Mode of preparation, estimation of dosage and forms of supply of vaccines derived from inactivated bacteria vary greatly. Five examples are given in Table I. RAETTIG *et al.* applied in their trials chiefly heat-killed corpuscular

Table I
Kinds of oral vaccines

Antigen	Method of production	Presentation	Source
<i>S. typhi-murium</i> <i>E. coli</i> enteritis	heat-killed corpuscular vaccine	fluid	RAETTIG <i>et al.</i>
<i>S. sonnei</i> <i>E. coli</i> enteritis	Boivin-extract	tablets	RAUSS <i>et al.</i>
<i>S. sonnei</i>	freeze-thawing method	granulate	GRAHNEIS <i>et al.</i>
<i>S. typhi</i>	formalin-heat-killed corpuscular vaccine	tablets	VEB SSW, Dresden
<i>E. coli</i> enteritis	sodium-desoxycholate extract	fluid	OCKLITZ <i>et al.</i>

bacterial suspensions. They found that heating at about 100°C for about 3–5 min was optimal. RAUSS, KÉTYI and their co-workers used trichloro-acetic acid extract according to BOIVIN. GRAHNEIS, THILO and others developed a special freezing-thawing technique for antigen preparation. A G.D.R. plant (VEB SSW) produces a formalin-heat-inactivated corpuscular vaccine against typhoid and paratyphoid fever.

We ourselves carried out trials with sodium desoxycholate extracts free of bacterial cells, obtained from *Escherichia coli* bacteria.

The efficacy of the vaccines mentioned has been demonstrated in animal experiments. There are, however, also some other techniques of preparation.

In previous trials we tested the following preparations: Corpuscular heat-killed antigen; corpuscular antigen inactivated at 100°C for 2 hr; desoxycholate extracts; cell walls prepared chemically; cell walls prepared mechanically; dodecyl extract.

In assessing the protective effect by the mortality rate of the animals against those of the controls we achieved the following results (Fig. 2). The best protective effect was obtained with mechanically prepared cell walls. Heat-killed corpuscular antigens afforded protection even if heated at 100°C

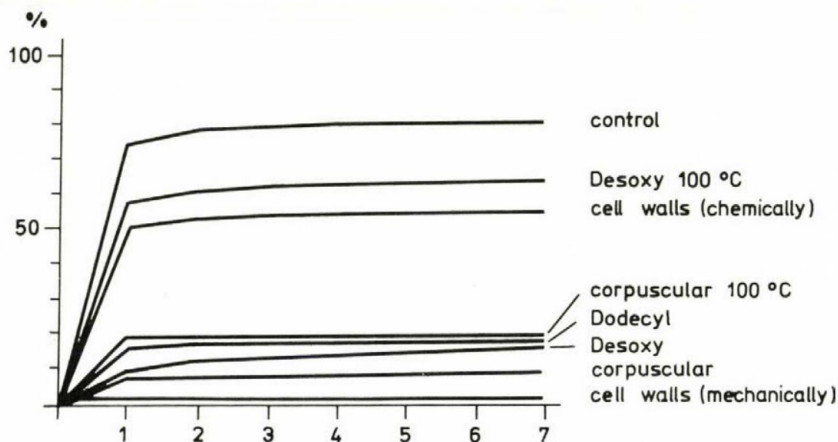


Fig. 2. Mortality rate of some preparations from enteritis-*E. coli* in comparison to control group

for 2 hrs. Sodium desoxycholate extracts and dodecyl extracts had similar protective effects. No protective effect was obtained with heated sodium desoxycholate extracts and chemically prepared cell walls.

BIRNSCHEIN *et al.* from the group of RAETTIG have reported recent studies on the immunogenicity of fractions of *Salmonella typhi-murium* in the mouse protective test. They tested whole cells, cell walls, protoplasm, RNA, lipopolysaccharide, lipid and protein. In these experiments, whole germ vaccines proved to be most effective. Nearly as effective were cell wall vaccines and cytoplasmic vaccines whereas chemically prepared fractions were slightly effective or ineffective. We do not want to analyze and discuss here these results but only draw the following conclusion.

Obviously, there are several methods to prepare inactivated vaccines promising a high efficacy in animal experiments. The actual protective substance in bacterial antigens has not yet been clearly defined. Hence, testing criteria and practical results of immunization will still have to prove the usefulness of the particular antigens or antigen fractions. And this brings us to the third question: What criteria are applied to test inactivated bacterial oral vaccines?

Like any vaccine, oral vaccines obtained from bacteria have to be tested for their harmlessness and tolerance as well as their efficacy before being widely em-

ployed. The testing for harmlessness of inactivated vaccines is a relatively simple procedure. Containing no live virulent organisms but only their fractions, there is no risk of their inducing the very infectious disease against which they are to immunize. Thus, the laboratory test is confined to demonstrating specific sterility and, in the case of fluid vaccines, it may be required that they are also unspecifically sterile. Oral tolerance can be demonstrated in animal trials and on adult volunteers. Since the degree of immunity of most of the vaccines is measured by intraperitoneal infection, it is reasonable to test the intraperitoneal tolerance of the vaccines. As suggested by THILO, the LD_{50} should be determined following the intraperitoneal injection of the vaccine in mice. This value should be at least 10 times lower than the LD_{50} of an equivalent number of reduplicable organisms of the production strains.

The problem in testing the efficacy of oral vaccines obtained from inactivated bacteria in animal experiments is the lack of an adequate model of intestinal infections in laboratory animals.

No experimental animal will contract typhoid fever, dysentery, cholera or *E. coli* enteritis following oral infection. Therefore many authors have chosen the active mouse protective test with oral immunization and atypical, *i.e.* intraperitoneal challenge. There are several variations of this test regarding dosage, rhythm of vaccination and challenge.

PIRINGER for example immunized mice 3 times within a period of 18 to 21 days and infected them 3 weeks after completing the immunization. RAETTIG reported a so-called basic trial of oral immunization. In this trial, mice in groups of 25 animals are immunized with the probe 10 times on successive days and challenged with two to three times the LD_{50} 7 days after the last immunization. As mentioned previously, efficacy is calculated by comparing the mortality of the immunized animals with that of the controls. This model has also been used by ourselves. RAUSS, KÉTYI and their colleagues carry out the active immunity trials in mice as a so-called "mucin test". Small experimental groups of 5 mice each are challenged with different graded oral doses of vaccines by means of the probe twice daily for three days in succession and then challenged with live organisms in 5% granular mucin. Besides the ED_{50} also the LD_{50} is determined in each case.

These are only three of many variations of this trial. The main difficulty is an accurate adjustment of the challenge dose because the virulence of the strains varies greatly. In our opinion the active protective test as practised by the RAUSS team is at present the best reproducible one.

The active mouse protective test assesses the protective effect of the orally immunized animal itself. In the passive mouse protective test, protective serum antibodies are demonstrated. This method has also been employed by several authors for testing oral vaccines (*e.g.* MOCHMANN, OCKLITZ *et al.*, RAUSS and KÉTYI). RAUSS and KÉTYI also use 10-day-old chicken embryos instead

of mice for demonstrating protective serum antibodies. The protecting serum is injected into a vein of the chorioallantoic membrane in graded dilutions and a dose of 0.1 ml. After a few hours the embryos are challenged intravenously with a suspension of live organisms. The rate of survival of the chicken embryos indicates the degree of passive immunity. The technique of this test is an extremely difficult one.

The question of humoral antibodies measurable by serological methods has played a great part in previous discussions on the efficacy of oral immunizing procedures. The fact that after oral immunization humoral antibodies are formed much less regularly than after parenteral administration of bacterial antigens, has been used for a long time as an argument against this method of vaccination.

In recent experimental work it has been found that approximately 100 times the dose of an antigen administered orally will produce the same level of humoral antibody titre as obtained after subcutaneous administration of the same antigen (RAETTIG and MARQUARDSEN, RAUSS and KÉTYI). Using more sensitive methods, some authors were successful in finding lower titres of humoral antibodies after oral administration. Thus for instance, THILO, HEMPEL and others detected by indirect haemagglutination a high proportion of serological conversion in children orally vaccinated against *Shigella sonnei*. We ourselves noted such seroconversion in new-borns immunized orally against *E. coli*. These infants were examined with the highly sensitive mixed haemadsorption technique according to FAGRAEUS. The view is general that the protective effect of oral immunization cannot be assessed by the level of the humoral antibody titres. Such seroconversions, however, indicate that the orally given antigen has brought about some immune-biological reaction and has not passed the gastro-intestinal tract without exerting an antigenic stimulus.

The testing criteria mentioned so far show general immunity or basic immunity after oral immunization but they are inadequate to detect local immunity. The procedures presented to achieve this are technically difficult or not easily reproducible and so hardly suitable for routine work. The simplest method yet is the demonstration of copro-antibodies in the faeces of orally immunized animals in the passive protective test as used successfully by the RAUSS team.

To measure the reaction of the intestine by the so-called "ligated gut" technique is a more difficult kind of trial. It was tested in both pigs and rabbits. The results are not easily reproducible. The procedure of the isolated intestinal loop described by us as a model of immunological examinations can be used for long-term studies of the reaction of an experimental intestine to local immunization. In a sucking piglet a portion of the intestine 50–70 cm in length is put out of action and infolded into the abdominal wall with an

oral and aboral stoma (Fig. 3). The small intestine is joined again by an end-to-end anastomosis and its continuity is re-established. Thus it is possible to continue feeding the animals normally during the experiment.

We were able to maintain such animals for more than five weeks. This method requires, however, intensive nursing and complicated technical apparatus.

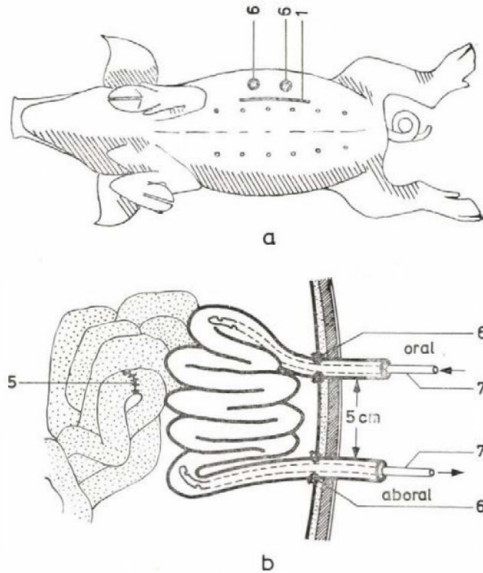


Fig. 3. Principle of the isolated intestinal loop (after HERING² et al.)

Demonstration of immunocompetent cells in the intestines was investigated by RAETTIG, LEVANON and ROSETINI. They applied the passive cutaneous anaphylactic test. Intestinal cells obtained from orally immunized animals were implanted intracutaneously in the abdominal skin of guinea pigs. After different periods, the homologous antigen was injected intravenously with Evans blue as an indicator. By this technique the authors succeeded in demonstrating reagin formation of the implanted cells.

Recently, WERNER has described a method for detecting immuno-competent cells in the intestines using the agar-plaque technique of JERNE, which will be discussed in more detail at this symposium.

In summary it may be stated that the testing criteria of oral, inactivated vaccines only provide evidence of their efficacy. They are, however, still inadequate to give a direct correlation between test result and anti-epidemic effectiveness. At this moment, anti-epidemic effectiveness can only be measured in clinical tests and field trials.

This brings us to the fourth question. What practical results have been obtained in the field of oral immunization with inactivated bacteria?

Since several papers of this symposium will deal with the vaccinations with inactivated bacteria, we only want to give a few examples regarding this question. The oral vaccines which have been used for the longest period are those applied against typhoid fever. Since 1921, over 1 million people have been immunized with these vaccines.

While in the trials performed during the first decades of this period primarily a fall in the mortality rate and a decrease in the number of complications were noted, an obvious fall in the morbidity rate has been demonstrated in recent years. The success, however, varied according to the vaccines and the dosage applied. The situation is similar as regards inactivated vaccines against shigella organisms. In this connection the question of basic and booster dosage is still open. Relatively few results are available of clinical tests of vaccines against *E. coli* enteritis. In a small field trial SCHREIBER obtained evidence of an anti-epidemic action. RAUSS's as well as our working team have achieved similar results which are to be presented in the course of this symposium.

On the whole, it can be stated that inactivated vaccines against various bacterial infections of the intestinal tract have proved their effectiveness in clinical testing. These tests, however, have to be continued.

And now we come to the last question: Which problems are left unanswered?

Over the last few years a great deal of information has been obtained on the mechanism of immunity of the intestinal tract. Remember, in particular, the studies on the role of IgA in intestinal immunity. Nevertheless, there are many unsolved problems.

In our opinion the following questions are of primary importance in oral immunization: (1) What is the adequate dosage of oral vaccines? (2) Which form of application is most satisfactory? (3) In which rhythm should the vaccination be carried out? (4) How often and at which intervals should a booster dose be given? (5) What are the best methods of measuring the local immunity obtained?

We are sure that at this symposium suggestions and answers will be offered to some of these questions.

Let us conclude by quoting ALEXANDER BESREDKA: "Taking account of the fact that natural infection induces the best immunity, artificial immunization has the task to follow those routes along which the pathogenic organism travels after entering the body. In short, immunization only means application of the old principle: 'natura non vincitur nisi parendo'."

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LIVE VACCINES AGAINST INFECTIONS WITH ENTEROBACTERIACEAE

PROBLEMS OF SELECTION OF ATTENUATED MUTANTS AND THEIR GENETIC STABILITY

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Summary. The report deals with the possibilities and problems of attenuation of *Enterobacteriaceae* by means of generation-limiting and non-limiting markers, the protective efficiency of such mutants for mice against lethal infections and the genetic stability of the attenuated strains.

The subject comprises two interlinked topics of discussion:

1. The possibilities of purposive attenuation by means of bacterial-genetic methods and the assessment of the degree of protection imparted by these mutants after single or repeated immunization.

2. Assessment of the stability of such mutants.

ad 1. (a) *Mutants with generation-limiting marker.* It is a well-known fact that streptomycin dependence, temperature sensitivity and purine auxotrophy under conditions *in vivo* will result in an interruption of growth. The mutation for streptomycin dependence is the one best known; it takes effect (just as the reversion for streptomycin non-dependence) with functionally modified ribosomal proteins [1–3]. In analogy to the influence of streptomycin on the translation of messenger RNA with streptomycin-sensitive strains [4], a correction of the false reading caused by the modified ribosomal protein [5–7] is carried out with streptomycin-dependent (Sm-d) mutants by means of the antibiotic. The Sm-non-dependent revertants [8] to be considered as suppressor mutants [9], still possess the Sm-d allele and, as so-called minus mutants, also show a reduced growth rate and are therefore overgrown by wild-type strains of the intestinal flora in the intestine and *in vitro*. In animal experiments, MEL *et al.* [10] proved that the shedding time of *Shigella flexneri* in mice is significantly reduced after immunization with a streptomycin-dependent *S. flexneri* vaccine.

In the settlement inhibition test (Fig. 1), our team showed significant differences in potency between the vaccines from living and heat-killed *Escherichia coli* associated with enteritis [11]. The mice orally immunized with the vaccine from living streptomycin-dependent bacteria after sterilizing the intestines with antibiotic did not settle the homologous streptomycin-resistant

strain in the intestine with but few exceptions, i.e., the faecal cultures did not show any growth of *Enterobacteriaceae* in most animals, and there only remained a carrier rate of about 10%. In controls and in mice which had been immunized with heat-inactivated antigen, the streptomycin-resistant strain could be cultivated from the faeces in about 90%.

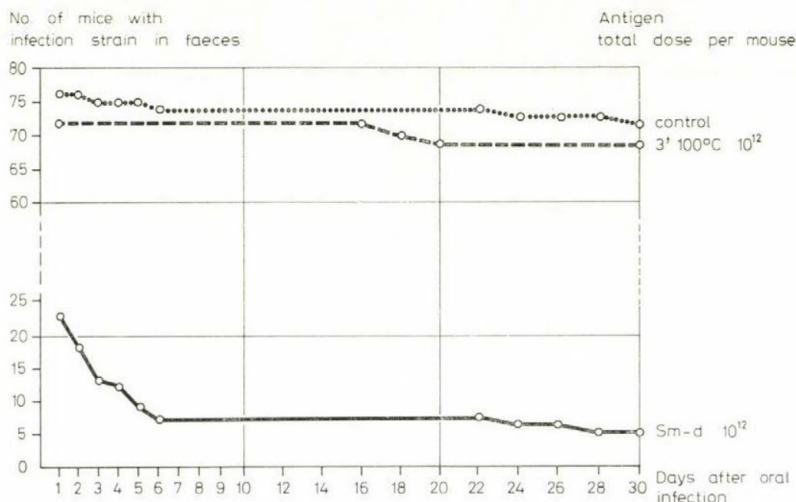


Fig. 1. Settling and shedding time of the homologous Sm-r *E. coli* strain in mice with streptomycin-"sterilized" intestine each as compared with 4×20 controls and with mice immunized orally with Sm-d or heat-killed O111:B4. Antigen dose 1st to 10th day per day and mouse 1×10^{11} cells. Oral infection on 20th day with 10^2 , 10^3 , 10^4 and 10^5 bacteria

The team of MEL [12] and the team of Metchnikoff Institute [13] reported that there were no complications in field experiments with Sm-d shigellae, but there was a significant protective action. In addition, our Soviet colleagues were able to prove, on the basis of histological studies, a desired limited residual virulence of the Sm-d shigellae [14]. The protective action of Sm-d enteritis- *E. coli* for apes has been reported by FELSENFELD *et al.* [15]. Vaccination incidents due to unstable vaccinating strains were not detected in any case. In extensive tests in volunteers, DU PONT *et al.* [16] succeeded only once in cultivating an avirulent *Shigella* revertant strain. We could also show the stability in man of Sm-d enteritis- *E. coli* and *S. sonnei* vaccinating strains (Table I) [17 and unpublished results].

When using Sm-d vaccinating strains it should, however, be borne in mind that changes in the O-antigenic structure of the Sm-d strains may occur with increasing culture passage frequency [18–21]; this is analogous to the fact that spontaneously avirulent strains frequently lose their immunogenic potency with continuous culture passages [22]. For the time being, the ques-

Table I

Oral application of 1.10^{12} Sm-d *E. coli* O111:B4 or O55:B5 or O26:B6 daily over a period of ten days to 30 adults and of 1.10^{11} Sm-d O111:B4 to 20 babies: shedding time of the vaccination strain, clinical side-effects and stability of the mutants *in vivo*

Test subject	Shedding after last antigen administration, days	Side effect	Wild-type or revertants in 21 days
Adult	1—3	Nil	Nil
Baby	1—3	Nil	Nil

tion cannot be answered as to how far streptomycin-dependent mutants find their way into the specific immunoprophylaxis of cyclic infections, since these strains only have a quantitatively and time-limited antigenic persistence of up to 20 days due to the deficient generation *in vivo*. Compared with inactivated antigens, however, streptomycin-dependent salmonellae impart an immunity lasting for 75 days from the tenth day after immunization (Fig. 2), the difference from the controls being significant only with live vaccines.

Temperature-sensitive (Ts) mutants as vaccinating strains also come under discussion. The inability of such microorganisms to grow at restrictive temperatures is caused by different mutations.

The block in DNA synthesis is due to a mutation at different gene sites. The mutation in the DNA E-locus results in a Ts DNA polymerase III [23]; in the DNA A-locus in the block at the beginning of DNA synthesis [24, 25]; and in the DNA B-locus in a temperature-dependent termination of DNA synthesis *via* primarily modified membrane proteins [25, 26].

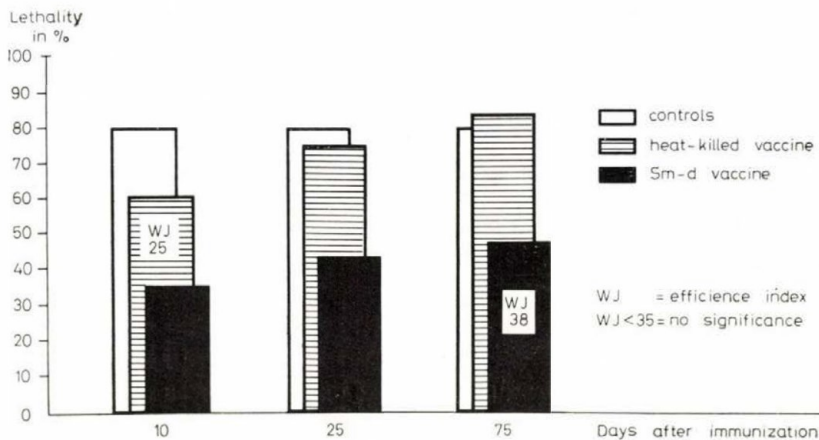


Fig. 2. Potency and duration of protective value imparted by killed and Sm-d *S. typhimurium* vaccine in mice. Oral immunization: 10^{11} cells daily for 10 days; oral infection: 10^9 wild-type strain cells on 10th, 25th and 75th days after immunization; period of observation: 18 days after infection

Due to RNA polymerases modified by mutation, there is an interruption of RNA synthesis and subsequent formation of protein above the limiting temperature.

In Ts mutants with deficient protein synthesis above the limiting temperature, the defect is conditioned also by different mutations. Certain mutants lack a factor which complements the ribosomal function [27] with restrictive temperatures in the cell-free supernatant, or the interruption of the RNA translation is disturbed by the terminator triplet UAA [28], or defined amino acid t-RNA synthetases do not function properly [29-31]. Another Ts mutant group shows a lysis of cells in the restrictive temperature range due to a defective synthesis of the mureine complex, where mainly the cross-linking peptide component is concerned [32-34]. Lysis of cells due to a cytoplasmic membrane defect with normal mureine complex is another possible cause of Ts [35].

The causes of Ts are to be found in structural changes caused by mutation, e.g. in a functionally defective 30 S-ribosome subunit [36], a base pair substitution in the t-RNA [37] or an amino acid substitution in the protein [38].

The kinetics of DNA, RNA and protein synthesis terminating in the case of a temperature shift are also of theoretical and practical interest. We differentiate between mutants in which DNA synthesis ends immediately after the temperature has increased to restrictive values, or continues for about another hour, with the RNA and protein synthesis continuing, as a rule, for a limited period [39-41]; and mutants which show a gradually reduced DNA synthesis in relation to the intermediate temperatures rising from 25°C to 37°C [40].

The limiting value for Ts and the stability of the Ts strains were determined by incubation of 10^8 mutant cells or of their logarithmic dilutions at 20, 25, 29, 32, 35, 37 and 41°C and by counting the colonies grown.

The results revealed three groups of mutants which, independently of the strain-characteristic limiting temperatures between 25 and 36°C, indicate an almost parallel decrease of colony frequency proportional to the increasing incubation temperatures above the Ts limiting value. Starting from the Ts limiting value, $T_{s\text{lim}}$, the influence of increasing temperatures on the number of colonies growing from 10^8 Ts germs was examined in the restrictive range. Six mutants each with phenotypically similar behaviour form a differential process group (Fig. 3): Group 1 — a curve descending sharply to zero; Group 2 — a curve descending sharply to 10^3 ; Group 3 — a relatively retarded curve but nevertheless continuously descending to zero.

Ts strains with immediate interruption of their metabolism in the restrictive range would correspond to the sharp descent of the curve. Ts strains with gradually decreasing metabolism or metabolism which is interrupted after an hour's incubation in the restrictive range, comply with the retarded con-

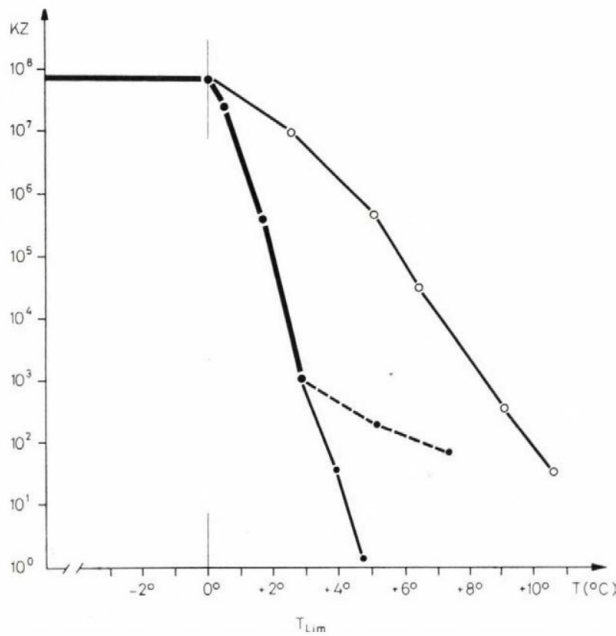


Fig. 3. Number of growing colonies related to 10^8 Ts cells in dependence on incubation temperature: summary of 6 mutants each with similar growth properties in groups with phenotypically similar behaviour after interpolation of the hypothetical mean critical limiting temperature ($T_{s\text{lim}}$) and comparison of the number of growing colonies with the incubation temperature elevated by 2, 4, 6 and 8°C

tinuous descent of the curve. The line bending off the sharp curve which increasingly levels off, is attributed to strains with a high reversion frequency.

Mutants of group 1 are a guarantee for optimum conditions with regard to their stability, since a limiting value of 33°C meets the requirement for lacking or negligible splitting off of revertants. Table II illustrates the virulence of Ts *S. typhi-murium* in dependence on limiting temperature and reversion frequency. With the exception of strain II whose restrictive range only starts at 36°C and which still shows 10^7 colonies at 37°C, all mutants are attenuated. As expected, only the slightly virulent strain offers protection against infection with 10^4 LD₉₅ with a single application of 10^5 mutant cells. The different revertant frequency of the various mutants has no influence on the behaviour of virulence in this test carried out with 10^5 cells as infectious dose. It is, however, to be expected that part of the revertants is also attenuated.

Purine auxotrophy is a growth limiting marker under conditions *in vivo*, since free purine metabolites are not available in the macroorganism. Thus, attenuation of the guanine-auxotrophic strains which are fully virulent in the presence of purine is based upon the stringent parenteral generation. Since strains which are reverted to prototrophy are again virulent [42-46], the atten-

Table II

Virulence of Ts mutants in dependence on limiting temperature and reversion frequency

<i>S. typhi-</i> <i>murium</i> Moscow	Optimum growth up to °C	10 ⁷ Revertants to 10 ⁸ cells		Virulence*	
		37°C	41°C	attenuated	protective value
II	36	7	0	(+)	+
I	35	1	0	+	—
III	35	2	1	+	—
IV	35	1	1	+	—
VI	35	3	2	+	—
VII	32	2	2	+	—
VIII	25	0	0	+	—

* 10⁵ mutant cells intraperitoneally per mouse; infection of surviving animals after 28 days with 10⁷ wild-type strain cells (LD₅₀ ≈ 1 cell)

uation of septicaemic salmonellae solely by means of the guanine auxotrophy marker is insufficient.

(b) *Mutants with generation non-limiting marker.* In contrast to the vaccinating-strain selection by means of generation-limiting markers, attenuation by means of generation non-limiting markers must begin with the biochemical-structural, enzymatic-toxicological and metabolic-physiological substrate of the virulence. Although our knowledge of the multiple causes leading to enteritis or septicaemia are limited with *Enterobacteriaceae*, there are many possible ways of isolating mutants of reduced virulence.

Salmonellae with a modified structure of the S-form O-antigens are attenuated due to the diminished protection against phagocytosis. In comparison with the wild-type strain, the smaller number of cells occurring *in vivo* is below the clinical threshold value, and the imparted protection is almost proportional to the residual growth in the macroorganism [47–49]. The range of such polysaccharide-defective mutants begins with strains of deficient cytidine-diphospho-abequose [50] and leaky mutants with only traces of the S-form “repeating units” and R-form strains, up to super rough form [49, 51, 52]. Genetic examinations have shown that the synthesis of the O-specific polysaccharides and of the basal oligosaccharides is determined by various gene sites lying wide apart from each other and designated *rfb* and *rfa* [53, 54]. The *rfa* locus which is close to the region for mannitol utilization contains the information for several sugar-transferring enzymes participating in the synthesis of the lipopolysaccharide-core region [55–57]. These R mutants have a defective core to which the synthesized O-specific hapten polysaccharides cannot be transferred. In contrast, the mutation in the *rfb* locus besides the his-

region brings about rough forms with complete core but missing O-specific side chains [55, 57-59].

Worth mentioning are in this connection those S-form mutants which have only about 50% of the S-form polysaccharides as compared with the wild-type strain. This quantitatively limited loss proved as a *S. typhi-murium* mutant of the O-antigen determinants 5, 12 and 4 is associated with a marked attenuation [60]. The cause of the low basic immunity against the homologous S-form wild-type strain achieved by immunization with R-forms [61-63], can be traced back to the same R antigen. This slight protection can be released against heterologous strains only with so-called Re mutants, since klebsiellae, *E. coli* and salmonellae are related only in the super rough antigen [64].

A special position amongst the cell wall defect mutants is held by strains in which the UDP-galactose-4-epimerase is missing due to mutation and which therefore grow as R-forms in normal cultures. While these so-called *gal E* mutants may incorporate the sugar into the S-specific polysaccharide chains with galactose being present, they cannot convert the UDP galactose, enriching to an excess into UDP dextrose. They form, therefore S-form polysaccharides with the galactose present *in vivo*, so that the good immunogenic potencies of such strains are conditional. Their attenuation is based upon the bacteriolysis induced by enrichment with UDP galactose [51, 65-67].

Table III

Differentiation of gal⁻ mutants which do not grow on synthetic media with 0.3% galactose

<i>S. typhi-murium</i> mutants	Phages P ₂₂ lysis			Galac- tolysis	Serological R-form			Mouse, i. p. 10 ⁶ cells	
	nutr. agar	synthetic media			nutr. agar	synthetic media		atten- uated	protec- tive value*
		gluc.	gal.			gluc.	gai.		
2	—	—	—	—	+	+	+	+	—
3, 4, 5	+	×	+	—	—	—	—	(+)	+
1, 8, 14	—	—	S	+	+	+	S—	+	(+)

* Infection on 28th day with 10⁷ wild-type strain cells (LD₅₀ ≥ 1 cell), S = single colonies grow, × growth after 48 hr

The *gal*-phenotypes mentioned in Table III which were selected simultaneously with the isolation of auxotrophy mutants because of deficient growth on synthetic medium with galactose, are interesting strains. Mutant 2 can be regarded as a simple R-form. A striking fact with the mutants 3, 4 and 5 is the P₂₂ phage lysis occurring only after 48 hr on synthetic medium with glucose, whereas the strains on nutrient agar showed a normal S-form behaviour. Despite the deficient galactolysis these strains are slightly attenuated. It appears that with this mutant phenotype the complete S-form on synthetic

medium without galactose develops with a certain delay. As regards galactolysis, rough form growth and P₂₂ resistance, strains 1, 8 and 14 correspond to the *gal E* phenotype on agar without galactose. Attenuation and limited protection fit into this schedule.

There are strains with reduced virulence among the amino-acid and vitamin-auxotrophic mutants. According to the literature, a certain percentage of the asparaginic acid, cysteine, histidine, glutaminic acid and PAB-auxotrophic mutants has a reduced virulence with *S. typhi* [42]. With *S. typhi-murium*, attenuated strains are to be found among the histidine, asparaginic acid, cysteine, methionine and glutaminic acid-auxotrophic mutants [46]. Relationships between growth intensity in the substituted synthetic medium and virulence did exist but only partially, and the different reversion rates to the prototrophy lying between 10⁻⁶ and 10⁻⁸ did not reveal any relationship to the differences in virulence. The conversion to prototrophy of the amino acid auxotrophy with attenuated strains by means of transduction did not show a uniform pattern, since strains with increased or even much more reduced virulence were found besides strains with unchanged degree of attenuation [46].

Table IV

Auxotrophy phenotypes: virulence of the mutants

Wild type strain *S. typhi-murium*: LD₅₀ $\geq$ 1 cell intraperitoneally per mouse. Mutant strain: infection dose, 10⁵ cells intraperitoneally (infection after 28 days with 10⁷ wild-type bacteria)

Phenotypes	No. of mutant strains			
	Σ	virulent	attenuated	attenuated with protection
<i>his</i> ⁻	48	41	6	1
<i>met</i> ⁻	23	20	3	0
<i>ser</i> ⁻	16	8	5	3
<i>tyr</i> ⁻	2	1	0	1
<i>hypo</i> ⁻	2	0	0	2
<i>ura</i> ⁻	7	5	1	1
<i>ade</i> ⁻	3	2	1	0
<i>gua</i> ⁻	7	6	1	0
	108	83	17	8

In Table IV we assigned the behaviour of the virulence of the mutant strains to the auxotrophy phenotypes examined. Attenuated mutants occurred only with certain phenotypes of *S. typhi-murium*. There are overlappings between the assignment of attenuated mutants to specific phenotypes, but they can be explained by the range of the mutants examined which were limited in

number. Agreement with regard to the auxotrophy phenotypes exists with histidine, methionine and the nucleic acid precursors. Worth mentioning is the discrepancy with guanine-auxotrophic strains. This marker is associated with a secure attenuation, whereas the reduction in virulence with our 7 strains was correct in a single case only. It is remarkable that part of the attenuated auxotrophy mutants should impart a pronounced protection. If these strains maintain their properties persistently, it would be possible to pre-select from this pool of mutants the strains for immunoprophylaxis or a coupling of markers. If the results obtained by us and those reported in the literature are interpreted according to the gene map of *S. typhi-murium* [68], the cause of the attenuation of part of the auxotrophy phenotypes could be the immediate vicinity of mutation to gene sites which control the building-up of the cell wall polysaccharides. The information for histidine biosynthesis is directly adjacent to the *rfb* locus; the same holds true for cysteine synthetase, serine transacetylase as well as orotic acid phosphorylase and the *rfa* gene cluster.

Another trend in attenuation is the hybridization of shigellae with *E. coli* [16, 69-74]. In this case certain gene parts of *S. flexneri* which are defined by gene mapping as "virulence genes" are exchanged against the corresponding section of the *E. coli* chromosome. Although virulent *S. flexneri* hybrids which have incorporated the xylose-rhamnose region of the *E. coli* chromosome are still able to penetrate into the intestinal epithelial cells, they can no longer cause disease. In addition, at least part of the regions is known on the gene maps available for shigellae and *E. coli*, which determine the functions for virulence behaviour, O-antigen expression [74-76] or defined enzyme function, e.g. in the sense of a delayed RNA synthesis [77], so that starting points for purposive attenuation exist. Similarly, systematic hybridizations between virulent and avirulent salmonellae bring about attenuated strains [78]. In general, new findings with regard to the enzymatic control of polysaccharide synthesis [79] are to be expected from the hybridization studies within the species salmonella, and conclusions with regard to the dependence of virulence on enzymatic efficiency and surface structures are anticipated from the formation of hybrids between salmonellae and other *Enterobacteriaceae* [80].

Attenuated strains are also found among the *uvr*⁻ strains [81-87], but until now a defined regularity has not been shown. Table V contains a summarized representation of the UV sensitive strains. We assumed that attenuated mutants were also present among these repair-defective strains. There is no relation between the quantitative degree of *uvr*⁻ and the behaviour of virulence. Sensitivity to X-rays, too, failed to point to some connection between these symptoms and attenuation. It was only the examination of our *uvr*⁻ mutants for sensibility to mutagens [88] carried out under another aspect,

Table V

Virulence and X-ray sensitivity of *uvr*⁻ *S. typhi-murium*. Mutants classified according to mutagen sensitivity

Mutants	Inhibition area diameter in mm		Attenuated ³	Protective value ⁴	UV sensitivity: rate of surviving, log. steps	X-ray test ⁵
	EMS 15 μ l ¹	DMS 5 μ l ²				
wild-type	13	30	—		0	C = X
Coli 3110 W	12	30			-1	
Coli 3110 <i>uvr</i> ⁻	30	57			-4	
24g	60	>90	+	+	-1	C $\approx$ X
17h, <i>pab</i> ⁻	58	>90	+	+	-1	C > X
23h	38	>90	+	+	-2	C $\approx$ X
6 strains	15-33	33-60	+	+	-4, -3, -2	C $\approx$ X
36g	38	60	(+)	+	-3	C $\approx$ X
3 strains	15-19	30-35	(+)	+	-4, -2	C = X
1 strain	26	58	$\emptyset$		-4	C $\approx$ X
4 strains	19-26	35-38	$\emptyset$		-4, -3, -2	C = X

¹ ethylmethanesulphonate

² dimethylsulphate

³ 10⁵ mutant cells intraperitoneally per mouse

⁴ infection of surviving animals on 28th day with 10⁷ wild-type strain bacteria (LD₅₀ >1 cell)

⁵ irradiation of micro-colonies with 6 kr: determination of difference in size to controls within 12 hr

which yielded useful criteria for pre-selection. It is possible that a coincidence exists between high mutagen sensitivity, X-ray sensitivity and relatively low UV sensitivity. These relationships must be compared and checked on the basis of more material. We were surprised by the observation that these mutants, to be regarded as attenuated, had a pronounced protective value.

In addition to the purposive attenuations mentioned, there are other possibilities of isolating strains of reduced virulence, in which the causes for attenuation are, however, largely unclear. This includes the isolation of mutants with low growth rate *in vitro* and *in vivo* such as the mutants of *Vibrio cholerae* [89] growing in cultures as dwarf colonies, or possibly of suppressor mutants derived from auxotrophic phenotypes. The same should apply to the selection of mutants adapted to unphysiological environments, when the strains have lost their virulence for the macroorganism as a result of a mutative adaption to the changed growth conditions, such as shigellae attenuated by letting them pass on nutrient agar repeatedly [22, 90, 91].

But it is assumed that the reduced growth rate *in vitro* of such shigellae [92] is hardly applicable to all attenuated strains.

ad 2. *Genetic stability of the attenuated mutants.* The use of attenuated mutants as vaccinating strains conditions their "genetic stability", i.e. back-mutation to the wild-type strain must not occur in the order of $\lesssim 10^{-8}$.

In Sm-d strains the stability of a mutant is characterized by two features [93]: revertant frequency to 10^8 Sm-d bacteria and the prolonged generation time of the revertants. We have never observed back-mutation to the wild-type strain in the growth curves obtained from revertants with the Sm-d enteritis- *E. coli* and *S. sonnei* strains examined by us, the reversion frequency of which were < 20 revertant colonies per 10^8 Sm-d bacteria.

Table VI
Revertant frequency per 10^8 Sm-d cells

Strain	Test series 1968	Test series 1969
O26:B6 (Lab) Sm-d 5	4	0.1
O55: B5 (Lab) Sm-d 8	27	7
O87: B7 (Lab) Sm-d 5	15	14
O111: B4 (2269) Sm-d 1	3	1

Table VI shows the reversion frequency of some Sm-d enteritis- *E. coli* over a period of two years. Selected mutants had split off from 10^8 cells only a limited number of streptomycin non-dependent revertants. As can be seen from Fig. 4 these strains now growing without streptomycin proved to be minus-mutants with a prolonged generation time. The residual attenuation of these streptomycin non-dependent revertants can furthermore be identified by the oxygen consumption of these mutants, LD₅₀ determinations and the overgrowth in mixed culture with wild-type strains of *Enterobacteriaceae* [93].

Conditions are similar with salmonellae. Table VII shows the stability of selected Sm-d salmonellae with an average reversion frequency of < 20 revertants per 10^8 Sm-d mutant cells. Revertants derived from these Sm-d mutants show, in comparison with the wild-type strain, a clearly prolonged generation time, but the sum of the averages of 20 revertants each shown in

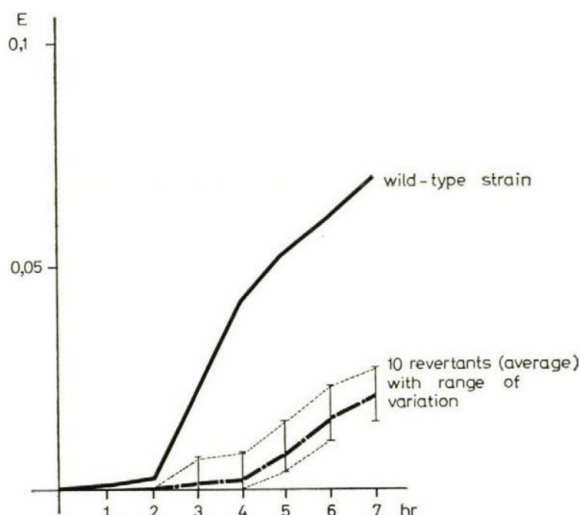


Fig. 4. Mean extinction (650 nm) of the nutrient broth culture of O111:B4 2269/66 and 10 Sm-non-dependent revertants

Table VII
Reversion frequency related to 10^8 Sm-d cells

Strain		S. dublin				S. typhi-murium		
		2a	3	5a	5b	1a	1b	2a
Range of variation	min.	3.5	1.0	>0.1	>0.1	>0.1	>0.1	0.7
	max.	39.8	40.8	0.3	2.2	6.0	20.1	6.9
Average from 13 test preparations		18.3	19.0	0.04	0.5	5.5	1.9	1.6

Fig. 5 includes strains with more or less reduced generation rate. It is quite obvious that the degree of attenuation of these revertants bears a certain relationship to the generation time of these strains. As can be seen from Table VIII, a complete attenuation exists for the Sm-d mutants, whereas the revertants indicate a residual virulence as a function of the gradually diminishing growth rate. Despite this residual virulence the surviving mice were protected only slightly against the high infection dose.

The results obtained by us with Sm-d salmonella mutants highly virulent for mice, agree well with the findings by MEL [94] who administered 10^{10} Sm-d *S. typhi* bacteria orally to more than 1000 children and adults and found that with selected strains the *in vivo* stability mediated by the streptomycin-dependence marker also applies to septicæmic salmonellae. All the laboratory tests and field experiments carried out with Sm-d mutants prove that — with

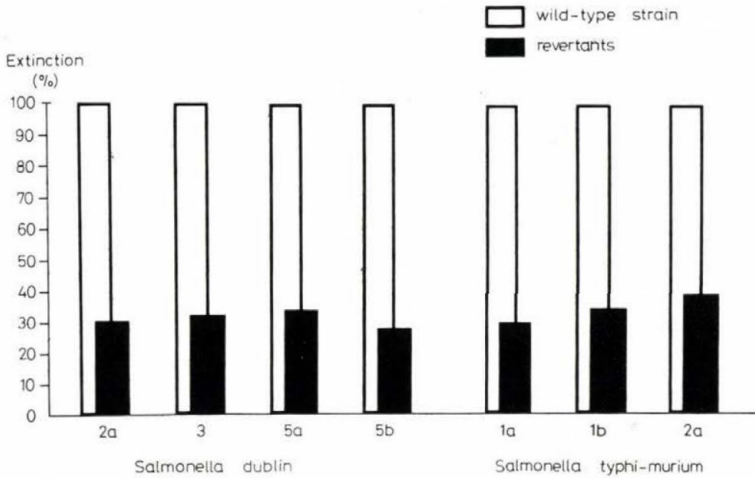


Fig. 5. Mean extinction (nutrient broth 3 hrs at 37°C) of 20 Sm-non-dependent revertants each, in comparison with the wild-type strain

Table VIII

Lethality of mice infected intraperitoneally with 10^5 mutant cells and protective value of this immunization when infecting the surviving animals with 10^7 cells of the homologous wild-type strain after 28 days

Mutants (<i>S. dublin</i> , <i>S. typhi-murium</i>)		Immunization with 10^6 cells i.p.		Infection with 10^7 cells i.p.	
		N	✱	N	✱
Sm-d strains		42	0	42	42
Revertants*	10—30%	126	9	117	110
	31—60%	84	35	49	40
	61—92%	42	28	14	8
Wild-type strain	<i>S. dublin</i>	54	51	—	—
	<i>S. typhi-murium</i>	180	177	—	—

* 3-hr growth value in comparison with wild-type strain

regard to infections occurring exclusively in the intestine — selected vaccinating strains possess the stability required, especially since the antagonistic potential of the adapted intestinal flora overgrows the revertants. It will have to be substantiated by further examinations whether this applied without restriction also to the same mutant type of pathogens of cyclic infections. Attenuation of guanine-auxotrophic strains which are fully virulent in the presence of guanine is based upon their restricted parenteral generation,

because no free purine metabolites are available in the macroorganism. Strains reverted to prototrophy usually prove to be virulent. Therefore, an attenuation of septicæmic salmonellae only by means of the *gua*⁻ marker is not sufficient which means that such mutants can be used as stable vaccinating strains only after the incorporation of an additional Sm-d marker [95].

In hybrid strains, a sufficient security against postvaccinal complications is closely linked with the coupling of two markers. *Shigella*-*E. coli* hybrids with deficient generation in the lamina propria and spontaneously avirulent *S. flexneri* mutants split off wild-type strains in the order of 10⁻⁸ to 10⁻⁹ [70, 72, 74]. It was only by the coupling of the two markers that postvaccinal complications could be eliminated. Similarly, a marker coupling will also be necessary for hybrid vaccinating strains against cyclic infections.

As to the stability of attenuated auxotrophy-phenotypes no information has been available. Although a direct coupling of auxotrophy and attenuation is obviously not worth discussing, we were searching for a correlation between reversion frequency to prototrophy and virulence behaviour in connection with the restoration of virulence in the mutation to guanine prototrophy. As can be seen from Table IX, there is no correlation between reversion

Table IX

Reversion frequency related to 10⁸ mutant cells and virulence of the auxotrophy phenotypes *ade*⁻, *gua*⁻, *hypo*⁻, *his*⁻, *met*⁻, *ser*⁻, *tyr*⁻, *ura*⁻

	>1000	100-1000	10-100	1-10	0.1-1	<0.1	Σ
Virulent	1	5	8	10	7	52	83
Attenuated	3	0	2	2	2	8	17
Attenuated protective value	1	0	0	0	3	4	8

Wild-type strain *S. typhi-murium* LD₅₀ ≈ 1 cell intraperitoneally per mouse; infection intraperitoneally with 10⁸ mutant cells; infection after 28 days intraperitoneally with 10⁷ wild-type cells.

frequency and residual virulence or detectable attenuation within the auxotrophy-phenotypes in which attenuated strains occur. In addition, it is obvious that no correlations exist between reversion frequency and an attenuation with or without protection with a single intraperitoneal application of 10⁵ mutant cells. Prolonged passages of these attenuated strains in both cultures and test animals will provide information on the stability of the attenuating marker. The same holds true for our attenuated UV sensitive strains.

The wide range of attenuating markers which have been defined by direct or indirect evidence, is either available as single marker for providing the vaccinating strain, such as streptomycin dependence, temperature sensi-

tivity, etc., or as a pool for a marker coupling, particularly for vaccinating strains against cyclic infections.

Marker coupling, however, raises new problems, since it is assumed that the immunogenic potencies of the vaccine are impaired at a too high attenuation. The question still remains as to whether the coupling of two growth-limiting markers such as streptomycin dependence and guanine auxotrophy or the coupling of a growth-limiting marker with a growth non-limiting marker are the optimum combination of a marker coupling (with regard to a desirable tissue persistence of the pathogens as a probable prerequisite for highly efficient vaccinating strains against cyclic infections). Further studies will show how far for instance the incorporation of a *rec⁻* marker [83, 84, 87] will itself cause an attenuation and, as second marker, will not interfere with immunogenicity. Finally I should like to express our conviction that the problems of attenuation, stability of the vaccinating strains, and marker coupling can be solved in the near future on the basis of consequent international co-operation in research work, which will produce highly efficient vaccines.

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SUBSTITUTION OF VIRULENT SHIGELLA AND ESCHERICHIA COLI VARIANTS BY AVIRULENT GERMS IN MIXED CULTURE

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Summary. It has been shown earlier that virulent *Shigella flexneri* and shigella-like *Escherichia coli* strains become avirulent for the guinea-pig eye and for the human intestine after 25—45 passages on 2% nutrient agar. The possibility of this substitution has now been studied in fluid medium. It was observed that the virulent strain *S. flexneri* 2a T₃₂ in mixed cultures with 7 different virulent *S. flexneri* strains and a virulent *S. sonnei* strain substitutes itself for the latter in a proportion of 86—100% for *S. flexneri* 2a, of 64—65% for *S. flexneri* 3a, of 79—99% for *S. flexneri* 4b, and of 70% for *S. sonnei*. Similar results were observed in the case of mixed paired cultures of avirulent shigella-like *E. coli* strains and different pathogenic *E. coli* strains (keratoconjunctivitis negative or salmonella-like strains).

Data in the literature [1—8] concerning the antagonistic or substitutive action of certain strains of the *Shigella* group by other dysentery bacilli species, or of *E. coli*, suggested the idea of studying the behaviour of avirulent and virulent *Shigella* or *Escherichia coli* strains in mixed cultures.

The present paper reports on results obtained by mixed seeding in fluid medium of certain virulent and avirulent variants of the homologous and heterologous species and serotypes of the microbial species mentioned.

The following *Shigella* and *E. coli* strains were used. (1) Strains virulent for the guinea-pig eye (KC⁺): *S. flexneri* 2a strains T₁, 328, 4622, 4605, 4643, *S. flexneri* 3a strain 188, *S. flexneri* 4b strain No. 6, *S. sonnei* strain No. 96, shigella-like pathogenic *E. coli* O124 : B17 3466. (2) Strains attenuated to avirulence for guinea pig eye (KC⁻): *S. flexneri* 2a T₃₂, *E. coli* O124 B17 3466 (28). (3) Salmonella-like pathogenic *E. coli* strains (KC⁻) [9]: *E. coli* O111 : B4, *E. coli* O111 : B4 M. B., *E. coli* O78.

A series of mixtures were obtained by seeding equal or different amounts of 24 hr broth cultures. These mixtures, regarded as mixed cultures, were always prepared with a *S. flexneri* KC⁺ strain and a *Shigella* KC⁻ strain or an *E. coli* O124 strain which has become KC⁻, and shigella-like pathogenic *E. coli* O124 or salmonella-like pathogenic *E. coli* strains.

The amount of initial inoculum necessary for obtaining these mixed cultures was 0.2 to 1.0 ml broth culture. The proportion of virulent (KC⁺ and salmonella-like) and avirulent (KC⁻) germs in each inoculum is shown in the tables.

Transfer from the mixed fluid cultures on plates with 2% nutrient agar was done after 24 and 48 hr incubation.

From the initial mixed culture, daily serial passages were likewise made in nutrient broth. Transfer from each of these broths was then done on plates with 2% nutrient agar after 24 and 48 hr incubation.

On the plates with transfers from the nutrient broth, the proportion of virulent and avirulent colonies was recorded, differentiating the colonies serologically by slide agglutination test or in the case of strains belonging to the same serotype, on the basis of colony morphology, the difference in agglutination with an absorbed type-specific serum, and the Serény keratoconjunctivitis test produced in the guinea pig.

The presence of eventual bacteriocins in the substituted strain was determined by diffusion in Petri dishes and inactivation with chloroform [10].

The presence of an eventual bacteriophage against the substituted strain was investigated by testing the lytic action of the chloroform inactivated of a 24 hr broth culture of the dominant strain against the substituted strain, at 37°C.

The differences in multiplication rate in broth of the strains investigated were determined in 1, 2, 3, 6, 9, 12 and 24 hr cultures with a "Spekol" spectrophotometer at 600 nm (performed in the Laboratory of Culture Media of the Dr. I. Cantacuzino Institute, Bucharest).

Table I gives the proportion of *Shigella* KC⁺ (virulent) and KC⁻ (avirulent) colonies obtained in mixed cultures at 24 or 48 hrs both in the initial mixture and in the subcultures performed daily.

Table I shows that the avirulent strain *S. flexneri* T₃₂ in mixed culture with the virulent *S. flexneri* strains T₁, 328, 188 and No. 6 or the virulent "S" *S. sonnei* strain No. 96, substitutes itself for the latter in a proportion of 86–100% for *S. flexneri* 2a; of 64–65% for *S. flexneri* 3a; of 79–99.7% for *S. flexneri* 4b; and of 70% for *S. sonnei*. Substitution occurs after 1 to 3 subcultures in nutrient broth, even when the inoculum is formed of 90% virulent and 10% avirulent germs.

Similar results, with a substitution of 100% of the virulent germs, were observed when the avirulent strain T₃₂ was cultivated in mixed culture with the virulent *S. flexneri* 2a strains 4622, 4643, and 4605.

In the case of mixed paired cultures with *E. coli* O124 : B17 3466 (28) KC⁻ and different pathogenic *E. coli* strains (KC⁺ or salmonella-like strains) there was likewise a substitution of the pathogenic strains by an *E. coli* KC⁻ strain (Table II).

From Table II it may be seen that starting from inoculations in which the avirulent *E. coli* O124 : B17 3466 (28) strain was cultivated in different proportions with pathogenic *E. coli* strains (the shigella-like *E. coli* O124 : B17 3466 strain or salmonella-like strains *E. coli* O111 : B4, *E. coli* O111 : B4 M. B., *E. coli* O78) mixed cultures were obtained in which a rapid substitution of the

Table I

The proportion of *Shigella* KC⁺ (virulent) and KC⁻ (avirulent) colonies obtained from mixed cultures (at 24 and 48 hrs) from the initial mixture and the daily subcultures

Mixed cultures	Virulence	Inoculum	Initial culture		Subcultures					
			24 hrs	48 hrs	1		2		3	
					24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	
<i>S. flexneri</i> 2a T ₃₂ +	a	50	98.5	99	100	100	100	100	100	
<i>S. flexneri</i> 2a T ₁	v	50	1.5	1	0	0	0	0	0	
idem	a	25	97	97	98	—	—	—	—	
	v	75	3	3	2	—	—	—	—	
idem	a	10	59	30	61	71	87	69	86	
	v	90	41	70	39	29	13	31	14	
<i>Sh. flexneri</i> 2a T ₃₂ +	a	50	49	70	88	78	90	100	98	
<i>Sh. flexneri</i> 2a 328	v	50	51	30	12	22	10	0	2	
idem	a	25	96	95	99.4	100	98.5	—	—	
	v	75	4	5	0.6	0	1.5	—	—	
idem	a	10	47	46	57	89	99	95	100	
	v	90	53	54	43	11	1	5	0	
<i>Sh. flexneri</i> 2a T ₃₂ +	a	50	67	59	54	67	36	—	65	
<i>Sh. flexneri</i> 3a 188	v	50	33	41	46	33	64	—	35	
idem	a	10	28	8	21	74	52	69	64	
	v	90	72	92	79	26	48	31	36	
<i>Sh. flexneri</i> 2a T ₃₂ +	a	50	93	58	92	82	95	94	99.7	
<i>Sh. flexneri</i> 4b Nr. 6	v	50	7	42	8	18	5	6	0.3	
idem	a	10	51	—	37	53	55	65	79	
	v	90	49	—	63	47	45	35	21	
<i>Sh. flexneri</i> 2a T ₃₂ +	a	25	89	73	—	75	70	—	—	
<i>Sh. sonnei</i> "S" 96	v	75	11	27	—	25	30	—	—	

a = KC⁻ strain (avirulent)

v = KC⁺ strain (virulent)

— = not counted

latter by the avirulent strain took place. The proportion of substitution varied between 95–99% with the *E. coli* O124 : B17 3466 strain, 81–98% with the *E. coli* O111 : K58 : B4 strain and between 68% and 63%, respectively, with the *E. coli* O111 : B4 M. B. and *E. coli* O78 strains.

As to the question to what extent the substituting effect of the avirulent KC⁻ variants was due to certain antibacterial factors of the bacteriocin or phage type, the following were observed.

Table II

The proportion of pathogenic *E. coli* and *E. coli* O124 KC⁻ (avirulent) colonies obtained from mixed cultures (at 24 and 48 hrs) from the initial mixture and the daily subcultures

Mixed cultures	Virulence	Inoculum	Initial culture		Subcultures					
					1		2		3	
			24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	
<i>E. coli</i> O124 3466(28) +	a	50	89	88	89	—	95	—	—	
<i>E. coli</i> O124 3466	v	50	11	12	11	—	5	—	—	
idem	a	90	97	99	99	—	—	—	—	
	v	10	3	1	1	—	—	—	—	
<i>E. coli</i> O124 3466(28) +	a	10	56	52	51	54	52	52	81	
<i>E. coli</i> O111 : (B4)	v	90	44	48	49	46	48	48	19	
idem	a	50	78	86	88	78	87	84	90	
	v	50	22	14	12	22	13	16	10	
idem	a	90	95	94	97	99	96	96	98	
	v	10	5	6	3	1	4	4	2	
<i>E. coli</i> O124 3466(28) +	a	50	70	69	62	71	68	—	—	
<i>E. coli</i> O111 M. B.	v	50	30	31	38	29	32	—	—	
<i>E. coli</i> O124 3466(28) +	a	50	49	49	56	57	63	—	—	
<i>E. coli</i> O78	v	50	51	51	44	43	37	—	—	

a = *E. coli* KC⁻ strain (avirulent)

v = *E. coli* pathogenic strain

— = not counted

In the substituting strains it was not possible to detect bacteriocins against any of the *Shigella* KC⁺ or pathogenic *E. coli* strains used in the mixed cultures.

No evidence was found of a bacteriophage lytic action of the inactivates of the avirulent strains against any of the substituted *Shigella* or *E. coli* strains. Although lysogenic, the avirulent *S. flexneri* 2a T₃₂ strain released lytic bacteriophage only occasionally and in small amounts which did not permit lysis of the substituted strains.

The multiplication rate of the germs in broth was determined spectrophotometrically at different intervals, from 1 up to 24 hr. The values recorded for both the *Shigella* and the *E. coli* strains showed that only in the case of the virulent *S. flexneri* 2a 4605 and *S. flexneri* 2a 4643 strains could a significant lag of growth be observed at 3 hr (0.035 for the virulent strains as against 0.095 for the avirulent strain) and at 24 hr only in the case of *S. flexneri* 2a 4643 strain (0.06 as against 0.185).

The results showed that apart from the antagonism between the *Shigella* and *E. coli* groups, reported by various authors [1, 5, 6], there also exists a possible substitution of some *Shigella* or pathogenic *E. coli* virulent strains by avirulent strains of the same species or the same group. This substitution takes place in 70–100%, even when the fluid media are seeded with an inoculum in which the avirulent strain represents not more than 10% of the virulent strain to be substituted.

Both with *Shigella* and with *E. coli*, substitution takes place within the same serotype and different serotypes. With the *Shigella* group, substitution may even take place when a different species is used (*S. sonnei*).

As regards the mechanism of substitution, an antagonistic action of the bacteriocin or bacteriophage has been excluded. An eventual increase in the multiplication rate of the avirulent germs was observed only occasionally and cannot be considered a determinant factor of substitution. Investigations concerning the existence of some undetected antagonistic principle or the impossibility of survival of virulent germs in subcultures on artificial nutrient media in the absence of a factor eventually existing in the host epithelial cell might perhaps supply an additional explanation of the mechanism of the phenomenon described.

Bearing in mind that the avirulent *S. flexneri* T₃₂ strain used by us for preparing a dysentery live vaccine possesses a marked substituting capacity for certain different *Shigella* serotypes or species, it seems possible that at the basis of the favourable preventive and therapeutical effect ensured by this vaccine [12–15] there may be, apart from the institution of a local immunity (proved experimentally [16, 17]), also a repression action exerted upon the wild strains circulating in the vaccinated community. This assumption is in agreement with recent epidemiological observations [11, 18] which showed that application of the live *S. flexneri* 2a dysentery vaccine leads to a marked diminution in the number of shigella carriers of different *Shigella* serotypes and species.

These data demand investigations into the local immunity against dysentery and into the favourable ecologic aspects of the application of the live dysentery vaccine.

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REQUIREMENTS FOR STERILITY TESTING OF ORAL BACTERIAL VACCINES

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Summary. The sterility requirements of oral enteric bacterial vaccines applied in the German Democratic Republic are discussed. It is suggested that the total number of contaminants should not exceed 1000/g of the final product; the number of fungi should not exceed 100/g. Higher numbers of germs are indicative of severe infringements of production hygiene; these must be avoided by permanent bacteriological control of the production process.

According to the requirements of every pharmacopoeia, preparations destined for parenteral use must be of extreme microbiological purity. Though control methods vary in the different countries, preparations are allowed to be applied parenterally only after their sterility has been proven. Pharmacopoeias likewise require sterility for ophthalmica. Sterility standards for preparations for external application are less strict [1–4]. USP XVIII, for example, requires absence of salmonella, coliforms, pseudomonas species as well as of pathogenic staphylococci, whereas in Switzerland and Belgium, for example, in addition to excluding the above-mentioned species, less than 100 germs/g are required [5]. For orally administered preparations – falling in the category of so-called non-sterile drugs – there is no conformity concerning microbiological purity [5–8]. Here the requirements to be met are dealt with rather generously, but few incidents are known which were caused by contaminated medicaments after oral application [3, 9]. The relative carelessness regarding a contamination of these preparations probably results from this fact. So USP XVIII merely requires the absence of salmonella and coliforms. In Belgium, the germ content has to be under 10 000 germs/g and in Switzerland the Food Act is to be considered. A number of countries such as England and the German Federal Republic [5, 10] are likewise lacking strict regulations. Sweden is an exception. After a severe incident – 200 people contracted a salmonella infection after taking tablets – it was ruled that preparations for oral use should contain less than 100 germs/g [9]. In Czechoslovakia the standards are strict, allowing 1000 germs/g.

Though in extensive testing of non-sterile preparations for oral use [3, 4, 6–8, 10–15] only a low number of ubiquitous germs was mostly found, norms for microbiological purity of oral drugs are generally required. The Food

Act stipulates the regulations demanding the absence of all potential pathogens in drinking water and foods, as well as fixing the admissible total number of germs [16, 17].

When determining the standards for the different kinds of oral vaccines used in this country the problem of contamination with heterogeneous germs should be solved as well. The following forms of oral bacterial vaccines for human use are currently produced in the G.D.R.: 1. extract vaccine, fluid; 2. inactivated whole-cell vaccines; dragées, granulates; 3. attenuated live vaccines, freeze-dried.

At the Symposium on Oral Vaccines held in Wernigerode in 1968, the question of sterility was also dealt with and some leads for quality standards were given [18]. It was demanded that inactivated vaccines be sterile. This requirement has been met with in the quality regulations. As regards sterility, the fluid oral extract vaccine complies with the preparations for injections; control is being effected according to DAB 7. At the same symposium it was suggested to allow up to 5000 apathogenic germs/g in oral vaccines.

Recently, referring to DAB 8 it is suggested that in case of non-sterile drugs, a total of up to 10 000 bacteria/g up to 100 fungi/g are acceptable on the condition that growth of the contaminants is prohibited in the final product [8, 19].

When fixing the quality standards for oral preparations, the Food Act is generally considered. In this connection we quote the example of Technical Quality Standard No. 25156 for the baby-food Instant, cereals for children on the basis of corn, which has been in force since January 1, 1972. According to this, 100 000 apathogenic germs/g, fungi and yeast below 100/g each, are allowed, and the absence of *Enterobacteriaceae* has to be demonstrated. There is no doubt that these rather generous requirements of the food industry can only serve as an orientation for oral vaccines.

We are faced with the task to fix the admissible total number of germs in an oral vaccine, which germs have to be eliminated at any rate, and to work out control methods. It is indispensable that large-scale bacterial cultivation and the preparation of the antigen should be done without contamination. This can be guaranteed solely by a proper production hygiene and strict control. In the final product the present technological level does not, however, exclude secondary contamination caused by microorganisms with any certainty.

In order to guarantee the innocuity required by the Medical Act (GBL I, No. 7 of May 28, 1964) the following species must be eliminated: 1. *Enterobacteriaceae*; 2. *Pseudomonas aeruginosa*; 3. *Staphylococcus aureus*.

In the case of inactivated oral vaccines in coated tablets we check for microbial purity first the antigen and subsequently the finished product. Both are tested in two stages.

1. Determination of the live germ content. Testing is done by adding the substance to melted agar and pouring into Petri dishes, using per test 100 mg antigen and 1000 mg dragées.

2. Elimination of the above-mentioned species by using the same amount of substance.

Testing of live vaccines made from streptomycin-dependent strains causes much difficulty. The usual methods – plating or membrane-filtration – cannot be applied in the presence of streptomycin-dependent germs. Based on our experiences, we therefore suggest to inoculate the surface of solid media in order to determine the total number of contaminants.

The following method was employed:

The contents of the ampoule is suspended in 2 ml of distilled water. Each Petri dish and/or each tube is inoculated with 0.1 ml of the vaccine suspension, as streptomycin adhering to the live bacteria allows a growth of dependent germs in some generations *in vitro*. The Petri dishes contain approximately 20 ml medium in a layer of 1 cm and each tube contains 10 ml broth. Apart from the media mentioned in Table I, in addition to each ampoule to be

Table I
Culture media used

Testing	Number of inoculated media per ampoule	Media	Media for further differentiation
Number of germs	3	Dextrose-agar	
Fungi	1	Sabouraud-agar	
<i>Pseudomonas aeruginosa</i>	3	Dextrose-broth	
<i>Staphylococcus aureus</i>	1	Dextrose-broth	Chapman agar
<i>Enterobacteriaceae</i>	1	Lactose-broth	Endo agar

tested 3 more dextrose-containing agar plates are inoculated with 0.1 ml of the distilled water used for suspension. These dishes serve to control the technique employed. All solid media are kept at 37°C for 48 hr and at room temperature for 72 hr. Then the lactose-broth culture is transferred onto Endo agar. The colonies growing here must be of the production strain, their determination being effected with specific anti-serum by slide agglutination. From the dextrose-broth culture, 0.1 ml is transferred after 48-hr incubation onto Chapman-agar at 37°C.

Colonies growing in a yellow colour are controlled by Gram staining and, in case of existing cocci, checked by the coagulase test. Finally, 3 glucose-broth tubes are controlled for the absence of *P. aeruginosa* by inoculation at 37°C for 48 hr and at room temperature for the same time. After this period a few drops of chloroform are added and the tubes are shaken. A green colour indicates a contamination with *P. aeruginosa*.

Calculation of the total number of contaminants is done on the basis of a comparison between the inoculated plates and the control plates.

According to DAB 7, as many ampoules are taken as the formula $0.4 \sqrt{n}$ indicates, where n = number of containers in the batch.

Number of ampoules to be tested	$0.4 \sqrt{n}$
Number of plates in given charge	$0.4 \sqrt{3n}$
Number of control plates	$0.4 \sqrt{3n}$
Sum of germs on all test plates	S_A
Sum of germs on all control plates	S_K
Mean number of germs per control plate	$\frac{S_K}{0.4 \sqrt{3n}}$
Mean number of germs per test plate	$\frac{S_A}{0.4 \sqrt{3n}}$

As one ampoule of our vaccine contains 200 mg substance in 2 ml, 10 mg are taken out in 0.1 ml and put on each plate; thus, the number of germs in 10 mg vaccine is:

$$\frac{S_A}{1.2 \sqrt{3n}} - \frac{S_K}{1.2 \sqrt{n}} \text{ or } \frac{S_A - S_K}{1.2 \sqrt{n}}$$

For 1000 mg ($\bar{y}$) the result is:

$$\frac{\bar{y} - 100(S_A - S_K)}{1.2 \sqrt{n}}$$

The charge meets requirements, if $\bar{y} \leq 1000$.

Controls made by both producers and state control authorities proved our production batches to be free from pathogens. A control batch was made to test the method. In doing so, the batch was exposed to contamination by atmospheric germs. Sterility controls of this batch showed ubiquitous germs, mainly aerobic sporogenic microorganisms, but also a few apathogenic staphylococci and fungi. Their total number in the different tests is shown in Table II.

Table II
Total number of organisms found in different tests

Tests	Number of ampoules tested	$\bar{y}$	Fungi
1	40	535	<10
2	21	506	0
3	40	487	<10

The differences are insignificant considering the range of errors in determining the number of germs [20].

We consider this method a reasonably safe and reproducible procedure and suitable for testing the microbial purity of live bacterial vaccines.

Based on our experience in the field of controlling inactivated vaccines, we are convinced that there must have been an infringement of the production hygiene to arrive at a limit of 5000 apathogenic contaminants/g recommended at Wernigerode in 1968. Testing more than 100 batches of oral inactivated bacterial vaccines we always found less than 1000 germs/g in the final product; pathogenic contaminants such as *Enterobacteriaceae*, *P. aeruginosa* and *Staphylococcus aureus* have never been detected.

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EFFICACY TESTING OF ORAL BACTERIAL VACCINES BY LOCALIZED HAEMOLYSIS IN GEL

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Summary. Localized haemolysis in gel allows the detection of specific antibody producing cells. The method has been applied for evaluation of the efficacy of oral bacterial vaccines. It is recommended to standardize the technique for routine laboratory control.

Detection of primary antibody response of the IgM type against sheep red blood cells by spleen cells obtained from immunized mice using the technique of localized haemolysis in gel was first applied by JERNE and NORDIN and JERNE *et al.* in 1963 [1, 2]. FRIEDMAN *et al.* [3] reported that antibody to the somatic antigens from Gram-negative bacteria are IgM rather than IgG globulins.

The technique of localized haemolysis in gel was modified to detect IgM antibody releasing cells to lipopolysaccharide antigens of *Enterobacteriaceae*.

As LANDY *et al.* [4], ESPOSITO [5], CHENG and TRENTIN [6] and others have shown, the method was considerably improved on coating the indicator erythrocytes with somatic polysaccharides of Gram-negative bacteria, since sensitized sheep erythrocytes participate efficiently in specific immune haemolysis.

Such modified red blood cells were used as the target for complement-mediated haemolysis in agar gel. The haemolysis appears in plaque surrounding the haemolytic antibody secreting cells [7].

We have modified the above technique for studying the immunogenic efficiency of an oral inactivated *Shigella sonnei* vaccine and now we are using for laboratory control this rapid and simple test instead of the conventional active mouse protection test.

The present paper reports on a study of specific antibody-forming spleen cells from mice immunized with a viable bacterial vaccine and from mice immunized with a bacterial extract vaccine.

As a model, we tested a viable streptomycin-dependent *Escherichia coli* vaccine and a sodium desoxycholate extract vaccine, both obtained from serotype O111 : K58.

Both vaccines are produced in our country, one by the Staatliches

Institut für Immunpräparate und Nährmedien, Berlin, and the other by the VEB Sächsisches Serumwerk, Dresden.

Experimental conditions were essentially similar to those applied in our first study [7]. Female albino mice of the strain AB, weighing 20 to 24 g, were immunized intraperitoneally with a single dose of 10^7 germs of the viable *E. coli* vaccine and an approximately equal dose of sodium desoxycholate extract. Calculation of the quantity of extract vaccine for immunization was performed according to MOCHMANN *et al.* [8].

The mice were sacrificed on the 4th day after immunization and from their spleen cell suspensions were prepared by teasing in Eagle's minimal medium. The number of nucleated cells per ml medium was estimated, without discriminating between lymphocytes, plasma cells and other forms of nucleated cells; 0.2 ml spleen cell suspension was added to 2.0 ml warm, melted Eagle-agarose-mixture to which had been added 0.2 ml of a 10% suspension of target sheep red blood cells (SRBC) sensitized with lipopolysaccharide (LPS) antigen derived from *E. coli* O111 : K58.

For coating SRBC with LPS the procedure described in [7] was applied. The mixture was carefully poured into Petri dishes 6 cm in diameter, containing a very thin base layer of 4% solidified agar gel. The base layer was filled into the Petri dishes on the previous day and dried within 2 hr at 120°C.

After incubation at 37°C for 1 hr, every plate was treated with 1.3 ml of guinea pig complement diluted 1 : 10, and then incubated for an additional hour. Within this time the plaques had become visible and were examined with a 6 × magnifying-glass. Subsequently, the number of haemolytic plaques per 10^6 nucleated spleen cells was determined.

Both vaccines were tested under the same conditions, each vaccine in 29 mice.

The method was controlled on (1) plates with agarose-Eagle medium and sensitized SRBC without spleen cells; (2) plates with agarose-Eagle medium, spleen cells from immunized mice and non-sensitized SRBC; (3) plates with agarose-Eagle medium with sensitized SRBC and spleen cells from non-immunized mice, to recognize the background forming units (BFU). These controls were carried out with complement. (4) A further control was performed without complement.

Table I shows the number of haemolytic plaques per million nucleated spleen cells of mice immunized with viable *E. coli* vaccine and with *E. coli* extract vaccine. The average number of plaques was obtained by testing 29 single spleens.

The number of plaques was higher with the extract vaccine than with the viable vaccine. However, with the extract vaccine the difference between the number of plaques detected in the original test and in the test with non-sensitized SRBC was very small.

Table I

Average number of plaques/10⁶ nucleated spleen cells of mice immunized with viable Sm-d *E. coli* vaccine and with *E. coli* sodium desoxycholate extract vaccine

Viable vaccine		Extract vaccine		BFU	Test without complement	Test without spleen cells
Original test	Test with non-sensitized SRBC	Original test	Test with non-sensitized SRBC			
9.0	2.4	17.7	15.4	1.6	—	—

The difference between the observations was not significant by both the F-test and the t-test. By the F-test the difference in testing the viable vaccine was significant: $cp < 1.0$.

The plaques detected on the control plates using non-sensitized SRBC might have been caused by haemolytic Forssman antibodies.

There seemed to be a close relationship between the antigenic mosaic of *E. coli* O111 : K58 and the antigenic determinants of SRBC. Also the antibodies to the *E. coli* extract vaccine appeared to interact in a higher degree with the antigenic determinants of SRBC than the antibodies to the viable *E. coli* vaccine.

The immunogenic efficiency of the sodium desoxycholate extract vaccine could not be proved by means of the localized haemolysis test. Either the method must be modified for this purpose, or other methods have to be applied.

FRIEDMAN *et al.* [3] compared the direct haemolytic plaque-method under the same conditions with a related but more direct antibody-plaque technique, utilizing viable *E. coli* as the target for antibody-forming cells secreting specific bacteriolysins described by SCHWARTZ and BRAUN [9], and the direct bacterial cytoadherence colony technique described by DIENER [10] for counting antibody forming cells to a purified protein antigen from *Salmonella*. This method was more sensitive than were the complement-dependent plaque methods.

The haemolytic plaque technique allowed to recognize IgM-forming cells in the intestinal tissue [11], and offered information concerning the other immune-globulins after oral antigen administration, above all the predominantly immune-globulin IgA in the lamina propria, for example with the indirect plaque-technique described by PIERCE *et al.* [12].

The methods of recognizing specific antibody producing cells to bacterial antigens may be more suitable than the conventional active mouse protection test for testing the immunogenic efficiency of oral vaccines. It is therefore recommended to use and standardize them for routine laboratory control.

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SELF-EXPERIMENTS WITH INGESTION OF VARIOUS BACTERIA

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Summary. Self-experiments with the oral ingestion of *Shigella*, shigelloid *Escherichia coli*, *Yersinia enterocolitica* and *Listeria monocytogenes*, and the conclusions drawn from the results concerning oral vaccination are summarized. Large-scale vaccination experiments and the testing of immunity developing after artificial infection of human volunteers with well-chosen strains and after careful organization are not considered too dangerous. At the same time, the necessity and lawfulness of such experiments are emphasized. The successful outcome of such experiments justifies the mass-scale application of effective and harmless oral vaccines. Due to its mass-scale, a vaccination experiment may improve the till now uncertain results for reducing the number of persons of full sensitivity.]

In the following, an account will be given of self-experiments performed mainly with enteric bacteria in order to provide a basis for oral enteral vaccination [1-4]. Since the results of experiments performed on a single subject cannot be generalized, they may be advantageous for comparison. The disturbing effects of the immunity and resistance presumably developed in the experimental subject may not have been significant due to the long intervals between experiments, especially as immunity against enteric bacteria is probably of a short duration.

Self-experiments with guinea pig eye negative shigellae. The first self-experiments were performed with *S. flexneri* strain 1b in 1955 [1]. Table I shows the virulence of this guinea pig eye negative strain.

Table I

Results of intraperitoneal inoculation of mice with guinea pig eye negative *S. flexneri* 1b strain

	Bacteria		
	Killed	Live	Live + mucin
LD ₅₀	4×10^9	4×10^8	$0.5-1 \times 10^2$

In Table II it is seen that bacteria killed by formalin or by heat treatment at 70°C induced equivocal clinical symptoms. Bacteria killed at 100°C were completely ineffective even in a single dose of 37 g, viz. a germ count of 10^{14} .

Table II

Oral self-experiments with Shigella flexneri

State of germs	Ingested bacteria		No. of intakes	Duration of experiment (month)	Clinical symptoms, WBC
	weight g	germ count			
Killed	47.0		7		±
70°C	15.5		17		±
100°C	24.0		2	2	—
100°C	37.0	10 ¹⁴	1		—
65°C stored for 12 days	7.0		1		+* 23 000
Live	0.5		4		—
	2.0		1	1	—
	10.0	2 × 10 ¹³	1		+* 18 000
	22.0	4 × 10 ¹³	1		+* 31 000
Total	165.0		35	3	
Live 1/2 year after last experiment		5 × 10 ¹⁰	1		—

* Intoxication

Note: 4 × 3 haemocultures negative

The first dose of live bacteria was 10⁸ germs. No symptoms were observed. The excreted germ count of *S. flexneri* was 2.5 × 10⁹, pointing to a propagation in the intestinal tract. The germ count excreted after a larger dose taken later was 5–10 times lower than the ingested, indicating that there was either no propagation or, else, its rate was surpassed by that of bacterial death.

After a dose of 2 g, no symptoms appeared. If a dose of 10 g or even of 22 g centrifuged (4.2 × 10¹³) live bacteria was taken, acute gastroenteritis or intoxication associated with nausea, headache, feebleness, shivers, epigastric pain and tenesmus developed in 2–3 hr. A leucocyte count of 31 000 was found at 5 hr. Then foamy thin faeces were expelled followed by rapid recovery in a few hours. What was the cause of the acute symptoms? Was it the enterotoxin [5–9] or some other biologically active material of live bacteria inactivated at 100°C, or perhaps the heat resistant endotoxin?

In contrast to most data in the literature [10–13], BIBINOVA *et al.* [14] found that high amounts of endotoxin were effective even when administered orally to guinea pigs. In our experiments, the 37 g bacterial mass containing a quantity of endotoxin decreased only by the heat effect was ineffective. On the contrary, data presented in the fifth row of Table II show that ingestion

of 7 g of bacteria killed at 65°C and stored for 12 days led to symptoms of intoxication with a leucocyte count of 23 000.

Ingestion of a high dose (30 g) of another strain, a guinea pig eye positive heat killed *S. flexneri* 2a strain, induced intractable vomiting for several hours. During this experiment I was so sick that no repetition or further laboratory tests could be performed.

Unfortunately, the bacterial suspension killed at 60°C was not completely sterile; it contained no living *Shigella* but some aerobic sporulated bacteria could still be detected in it.

The acute symptoms mentioned to develop after the intake of 22 g live *S. flexneri* 1b disappeared in 8–10 hr, but tenesmus and the expulsion of some mucous faeces occurring once daily persisted for 4–5 days. Full recovery took 5–6 weeks. Repeated tests revealed shigella excretion for 4 days at most. *S. flexneri* isolated from the faeces failed to induce inflammation when introduced in high amounts into guinea pig eyes. The 12 haemocultures prepared after the intake of bacteria were all negative.

My last experiment with guinea pig eye negative *S. flexneri* 1b consisted of the ingestion of 5×10^{10} live bacteria after a half year interval. Such an interval seemed necessary for excluding the effects of non-specific resistance and specific immunity that might have developed as a consequence of the repeated intakes of bacteria. After this last trial, no symptoms whatever appeared. It has been learnt since that the doses of live shigellae used for vaccination are of this magnitude.

Bacteria taken serially by mouth led to an insignificant increase in the agglutination titre in the serum of experimental persons. In passive immunization experiments in mice, 0.1 ml of the normal serum of the experimental person, 0.005 ml of serum obtained after the intake of killed bacteria, and 0.007 ml of serum obtained after the intake of killed and live bacteria protected half of the animals against about 75 LD₅₀ (Table III).

Coproantibodies were detected in small amount. Due to the magnitude of the different doses and the chronic intake of bacteria, my data may be interesting from the aspect of oral vaccination with live *S. flexneri*. The dose recently used for vaccination with live shigellae contains 10^{11} bacteria [15]. The dose of *S. flexneri* 1b causing acute symptoms in my experiments was several hundred times higher.

Experiments with six S. sonnei II strains and a S. sonnei I strain. Of the data obtained with these strains [1, 3] only some surprising ones will be discussed. The *S. sonnei* phase I strain used was negative in the guinea pig eye test whereas one single germ of it killed the mice when introduced intraperitoneally together with mucin.

Table V shows that neither *S. sonnei* II nor the already mentioned *S. sonnei* I strain caused symptoms even when taken in 10 g doses and their

Table III
Passive immunization experiments in mice

Dose	Control without serum	Serum samples of experimental person								
		Normal			After intake of killed bacteria			After intake of killed + live bacteria		
		0.1	0.01 ml	0.001	0.1	0.01 ml	0.001	0.1	0.01 ml	0.001
7.5×10^6	0/4									
1.5×10^6					6/6	4/6	0/6	5/6	5/6	1/6
7.5×10^5	0/4									
1.5×10^5		4/6	2/6	1/6	5/6	6/6	0/6	2/6	2/6	1/6
7.5×10^4	0/4									
1.5×10^4		4/6	4/6	1/6	5/6	6/6	1/6	5/6	5/6	0/6
7.5×10^3	1/4									
7.5×10^2	3/4									
ml serum neutralizing 75 LD ₅₀		0.01			0.005			0.007		

Note: serum was injected 45 minutes before infection

Table IV
Data of experimental strains

No.	Strain	Origin	Guinea pig eye reaction	Mucin technique, intraperitoneal mouse LD ₅₀
1.	<i>S. sonnei</i> II	K. MB.	negative	—
2.	<i>S. sonnei</i> II	Sz. MB.	negative	—
3.	<i>S. sonnei</i> II	B.MB.	negative	—
4.	<i>S. sonnei</i> II	Szi. MB.	negative	—
5.	<i>S. sonnei</i> II	R. MB.	negative	—
6.	<i>S. sonnei</i> II	R. "5"MB.	negative	—
7.	<i>S. sonnei</i> I	1942	negative	1 germ

isolation from faeces either failed or was successful for 1—2 days only. During 2 months, I have ingested 42 g of bacteria without developing symptoms.

Notes to the above experiments. In view of my marked hypacidity, a neutralization of the gastric contents seemed unnecessary. From the faeces a dilution series was prepared and the germ count was determined in each dilution by surface plating on 4 different media. Strains resistant to antibiotics isolated from the faeces have not been studied. Since by means of the method

Table V

Results of human oral self-experiments with live Shigella sonnei strains

Strains	Ingested bacteria		No. of intakes	Duration of experiment (month)	Symptoms
	weight, g	germ count			
<i>S. sonnei</i> II	6.5	1.3×10^{13}	6	2	—
Mixture of 5	11.0	2.0×10^{13}	1		—
<i>S. sonnei</i> II	2.5	5.0×10^{12}	3		—
<i>S. sonnei</i> I	6.5	1.0×10^{13}	10	1	—
	9.0	1.8×10^{13}	1		—
	6.5	1.3×10^{13}	1		—
Total	42.0		22	3	

applied shigellae could be isolated from the faeces for 1–2 days or 4 days at the most, it was assumed that no bacterial centres had developed after the intake of non-virulent bacteria. This assumption has since been confirmed by several experiments. Among the shigellae isolated from the faeces after oral intake none was found to have assumed guinea pig virulence even after the intake of nearly 100 g live bacteria. No laboratory test yielded evidence of the activity of bacteria or of an increase of their virulence and only mild clinical symptoms indicated that the high doses taken were not indifferent for my organism. The symptoms might have been caused by any acute or chronic toxic injury. The total quantity of live and killed bacteria I ingested during 6 months amounted to 205 g.

It is known that specific or group specific immunity can be induced if the same mucous membrane, *e.g.* the guinea pig conjunctiva is used for both immunization and testing [16, 17]. On the other hand, it is undecided whether after specific immunization it is possible to detect the immunity from the direction of another mucous membrane, *e.g.* conjunctiva, intestine. Studies of this problem have been presented at this symposium. It is another question, how many aspecific components are involved in specific immunization especially if the immunization process induces an inflammatory change. After inflammation of the conjunctiva and cornea induced by NaOH solution, a low level of immunity was observed to develop [18] following conjunctival infection (aspecific tissue immunity).

In my experiments performed with a heterologous bacterium in 1952–55, a millionfold increase in aspecific immunity and 50-fold increase in the resistance against endotoxins were obtained after repeated endotoxin treatment of mice [19, 20]. (Table VI).

Table VI

Aspecific antibacterial immunity developed after several intraperitoneal treatments

Pretreatment	<i>E. coli</i> 055 in mucin intraperitoneally											
	1×10^{10}	5×10^9	5×10^8	5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2	5×10^1	5	1-2
Mice repeatedly treated with <i>S. flexneri</i> and with <i>S. sonnei</i> endotoxin	0/2	0/3	1/3	3/3	2/2	3/3	2/2	3/3	3/3	—	—	—
Untreated mice	—	—	—	—	0/2	0/3	0/3	0/4	3/4	2/4	2/4	4/4

Resistance against endotoxins developed after several intraperitoneal infections

	<i>S. flexneri</i> endotoxin, ml												
	0.5	0.4	0.3	0.2	0.15	0.05	0.04	0.03	0.02	0.017	0.014	0.01	0.008
Mice repeatedly treated with <i>S. flexneri</i> endotoxin	1/2	3/4	4/4	5/5	5/5	—	—	—	—	—	—	—	—
Untreated mice	—	—	—	—	—	0/10	2/10	0/10	1/22	2/5	4/10	9/17	11/15

Is it possible to attain aspecific immunity by testing the site of local immunization from the direction of mucous membranes? Studies to answer this question are in progress in our laboratory.

For the oral immunization, high doses of bacteria have recently been used. These doses alone are harmless, yet in the case of simultaneous oral vaccination with various bacteria the high number of bacteria may elicit symptoms. In this respect, the studies performed by RAETTIG [21] and MARQUARDSEN [22] seem interesting. These authors have shown that after simultaneous oral immunization with several antigens, the level of immunity developed against each of the components is about the same as that obtained after a similar sized monovalent vaccination. It could be hoped for the elaboration of a harmless polyvalent oral vaccination that immunity would develop not only against infections detectable by intraperitoneal testing but also against those occurring from the direction of the mucous membranes.

Daily live oral vaccination or giving bacteria or antigens mixed in commercial salt, owing to the wide consumption of salt, would be an ideal oral vaccination. My data offer a certain basis to answer the question whether live or killed bacteria or endotoxins or other toxic bacterial materials may cause any harm after chronic oral intake.

Experiments with shigelloid E. coli. Experiments performed in 1955–56 have shown that infection of mice by the intraperitoneal mucin technique is not analogous with human dysentery infection. Human infections show a parallelism with the guinea pig eye reaction [1, 3]. In my experience, each bacterial pathogen causing a dysentery-like change in the human colon also induces a guinea pig eye reaction. On the basis of this assumption have I obtained my first guinea pig positive *E. coli* strains, the first representatives of the shigelloid group. I intend to discuss these strains as I have performed self-experiments with them, and the problem of these bacteria fits well into the topics of the present symposium due to their close immunogenic relationship with shigella.

A serious water-borne enterocolitis epidemic affecting 30 000 persons and causing disease in 10 000 among them occurred in the town of Veszprém in 1958. The causative agent was *E. coli* O124 [23] and, as expected, it proved guinea pig eye positive. In the same year, a new and even more typical *E. coli* strain was isolated in pure culture from a febrile patient suffering from enterocolitis [3]. This strain, termed by me *E. coli* Öskü [3], later proved to be *E. coli* O143 [24] and was also guinea pig eye positive. These strains were the first typical eye positive *E. coli* strains from the enteropathogenic group termed by us shigelloid [25] and later called invasive by FORMAL *et al.* [26].

Since then this group has gained more and more members. The strains listed in the first row of Table VII are considered shigella by some authors [27, 28] but – in a strict biochemical sense – *E. coli* cultures may also be classified into this group.

Table VII
Guinea pig eye positive E. coli strains

1.	Strains closely related to <i>Shigella</i>
2.	<i>E. coli</i> O18a, b : K76*
3.	<i>E. coli</i> O28a, c : K73
4.	<i>E. coli</i> O29*
5.	<i>E. coli</i> O32
6.	<i>E. coli</i> O112a, c : K66
7.	<i>E. coli</i> O115
8.	<i>E. coli</i> O124 : K72*
9.	<i>E. coli</i> O136 : K78
10.	<i>E. coli</i> O143 : K? : 1 (Öskü)*
11.	<i>E. coli</i> O144 K?2
12.	<i>E. coli</i> ? to be determined*
13.	<i>E. coli</i> of RUCHMANN and DODD [30]

* Guinea pig eye positivity shown first in this laboratory
(018 strains are usually negative)

Of the *E. coli* strains shown in the 2nd—12th rows of Table VII, each, except the 11th *E. coli* O144, could be found in our laboratory studies. Furthermore, the positive guinea pig eye reaction of strains 2, 4, 8, 10, and 12 has been demonstrated in our laboratory [29].

The old strain of RUCHMANN and DODD [30] causing enterocolitis in man and keratoconjunctivitis in the scarified rabbit eye may also be listed with this group even though as an old laboratory strain it fails to give a positive eye reaction now.

In contrast to the guinea pig eye positive *E. coli* strains listed above, the failure of *E. coli* strains associated with infantile diarrhoea to induce an eye reaction has been known since the first paper of SERÉNY [31]. Based on the above data, a classification and the extrapolated characteristics of human pathogenic *E. coli* strains were presented in 1964 (Table VIII) [25].

STENZEL published a similar classification for the separation of *E. coli* dyspepsiae and shigelloid *E. coli* [32]. NOVGORODSKAYA when attending the 4th Hungarian Congress of Microbiology in 1964, adopted our classification, and called attention to the connection between *E. coli* dyspepsiae and the *Salmonella* group. This author had an important role in the popularization of this classification of enteropathogenic *E. coli* [33, 34], which has gained acceptance all over the world [26, 35]. The introduction of further biomodels (mouse lung, murine vagina, murine intestinal tract, cell cultures), genetic, microscopic, and ultrastructural studies as well as vaccination experiments have contributed to an improvement of this classification [36]. The chemical

Table VIII

Characteristic features of human enteropathogenic E. coli strains (1964)

	<i>E. coli</i> (infantile enteritis)	<i>E. coli</i> (shigelloid)
Bacteriology		
Bacteria in duodenum	+	—
Guinea pig eye reaction	—	+
Seroidentity with <i>Shigella</i>	—	+
Genetic connection with <i>Shigella</i>	—	+
Pathology	Small intestinal involvement	Colonic involvement, ulcers
Epidemiology		
Nursery epidemics	+	—
Spreading in nursery	+	—
Water and food borne epidemics	—, ± [59]	+
Spreading by contact	+	±
Clinical picture	Dyspepsia	Like shigellosis
Age	Mostly infants	Adults and children
Parallel reaction of guinea pig eye and human colon	—	+

differences between the two groups have been revealed by immunoelectrophoretic analysis of the antigens [37]. *E. coli* dyspepsiae strains have a cathodic O and a K moiety probably within the same molecule, while shigelloid *E. coli* contains similar but anodic antigens.

There are several data [17, 38] suggesting that these strains are related immunologically with each other and with shigellae. Immunity produced by one of them results in some immunity against the other; this may be a result of a specific and aspecific (tissue) reaction or a consequence of a common mechanism of action. Nevertheless, it induces the hope that immunization against shigellae may ensure protection against shigelloid *E. coli* as well [17, 38]. The significance of an eventual vaccination against shigelloid *E. coli* depends on the frequency of such strains in the different countries.

E. coli O124 occurs with a high frequency (1–2%) in Hungary. It is almost as frequent as shigellae. *E. coli* O143 has a frequency of about 0.1%. In that part of the Hungarian gipsy population which lives under special and adverse hygienic conditions, the ratio of *E. coli* O124 and O143 positive patients is as high as 10–20% and 1–2%, respectively [39]. *E. coli* O143 also caused a water-borne epidemic. Other shigelloid *E. coli* strains are much less frequent in Hungary.

Table IX

Origin and serogroup of some cultured guinea pig eye positive E. coli strains

Origin	No. of examined faeces	No. of strains	<i>E. coli</i> O group
Monkeys	425	5	O18a, c
		5	O28a, c
		1	O115
		1	O124
		2	O143
Foreign students	60	1	O18a, c
		1	O28a, c
		3	O29
		1	O136
		1	O143
Gipsies	360	35	O124
		15	O143
		1	O115
		1	?
People under poor hygienic conditions	200	9	O124
		2	O115
Water-borne epidemic	60	13	O143
Laboratory worker		1	O115
Routine stool examinations	15 000	165	O124
		9	O143

Shigelloid *E. coli* was frequent in students coming from tropical countries; from 60 faecal samples 6 were positive. Monkeys imported from India are also frequently carrying these pathogens.

Under adverse hygienic conditions, not only a more rapid spread but also a continuous *de novo* formation of shigelloid *E. coli* may be surmised (hybridization?).

Our inoculating into the guinea pig eye of mixed primo-cultures from faeces [3] allowed to isolate more strains in our laboratory than in others. Still, these strains are frequent also in other countries, *e.g.* in Japan and Brasil [40]. Yet, their frequency is low when compared to the high number of enterocolitis occurring in the adult population. On the other hand, in connection with acute enterocolitis and water-borne epidemics not only guinea pig eye positive *E. coli*

strains but also those with other characteristics must be considered pathogenic. This necessitated the formation of a special group of unknown strains. In 1970, I had the opportunity to study *E. coli* O148: H28, a strain causing enterocolitis in a British military group; it gave a negative eye reaction.

Table X

Groups of human enteropathogenic E. coli and Shigella

In 1964 (RÉDEY)					
	<i>E. coli</i> infantile enteritis	<i>E. coli</i> "others"	<i>E. coli</i> shigelloid	Shigellae	
Guinea pig eye reaction	—	—	+	+	

In 1969 (FORMAL)					
	<i>E. coli</i> infantile enteritis	<i>E. coli</i> pathogenic by enterotoxin	<i>E. coli</i> "others"	<i>E. coli</i> shigelloid	Shigellae
Guinea pig eye reaction	—	—	—	+	+
Enterotoxin effect	?	+	—	—, ?	?, +
Rabbit ileal loop		+	—		
Example	O55, O111	O6, O148		O124, O143	

A similar strain and strain O6:K?:H16 causing human colitis on the guinea pig eye were studied in human volunteers and on the rabbit intestinal loop [41]. Both strains were found pathogenic for man and the rabbit intestinal loop and to produce enterotoxin. Thus a new group characterized by eye negativity and enterotoxin positivity was separated from the group of unknown pathomechanism.

Neither the frequency nor the significance of this enterotoxin positive *E. coli* of human and animal pathogenicity can be fully assessed. The studies of GEMSKI *et al.* [42] showed that the enterotoxin produced by shigellae and shigelloid *E. coli* [43, 44] does not play an important role in human dysentery. The enterotoxin may have more significance in the pathomechanism of *E. coli* dyspepsiae. Enteritis and duodenal processes caused by these latter kinds of *E. coli* are similar to the so-called cholera nostras [45].

Virulence studies by self-experiments. I have already mentioned my self-experiments with guinea pig eye positive shigelloid *E. coli* O143, to obtain evidence of its human pathogenicity. I have attempted to reach a small but certainly infectious dose instead of the minimum dose. A dose of 10^9 germs was therefore used which caused a fever of 39°C and enterocolitis with blood in the faeces. The parallelism between the eye reaction and the reac-

tion of the human intestinal mucosa was thus proved not only in a negative way, *viz.* with avirulent *shigella*, but also positively with virulent shigelloid *E. coli* [3]. It should be noted that three types of shigelloid *E. coli* caused laboratory infections leading to enterocolitis only because the workers did not believe in the pathogenicity of those strains.

In the experiments performed thus far, no efforts have been made to estimate the minimal infectious doses. It is obvious, however, that the doses described by SHAUGHNESSY *et al.* [46] as well as those of 10^9 germs applied by us were too high as compared to those causing natural human infection. In an attempt to find strains more virulent than the average, two methods were used. (1) From shigelloid *E. coli* strains isolated from routine material, an arbitrarily estimated 20 000 germs was used for human infection. Of the strains tested, an *E. coli* O124 strain was found [47] which in a dose of 2×10^4 caused enterocolitis accompanied by fever and mild clinical symptoms, as shown in Table XI. Its dose inducing guinea pig keratoconjunctivitis was 10^4 . (2) Assuming that the parallel reaction of the human intestine and the guinea pig eye was not

Table XI

Infective doses of enteric pathogens in human experiments

Group	Infective doses		Symptoms	Guinea pig eye reaction
	others	own		
<i>E. coli</i> (infantile enteritis)	$10^7 - 10^9$			
<i>E. coli</i> (shigelloid)				
O124	5×10^5	$2 \times 10^4 \pm$	37.8°C (1963)	$+ 10^4$
O143 (Öskü)		$10^9 + + +$	Blood in stools, 39°C (1958)	$+ 10^5$
<i>S. flexneri</i>	$10^9, 10^{11}$ [46]			$+ 10^5 - 10^7$ $+ 5 \times 10^2$
2a		2×10^3	Mild symptoms	
2a	$10^4, 10^5$ (FORMAL, 1969)			
2a		2×10^5	Superinfection. Blood in stools, 39.2°C (1964)	
<i>Y. enterocolitica</i>		$3 \times 10^8 +$ $2 \times 10^9 + +$	Mild starting Superinfection. Acute, subacute diarrhoea, 38°C (1965)	$+ conjunctivitis$ $+ conjunctivitis$
<i>L. monocytogenes</i>		10^{11}	— (1971)	—

only qualitative but also quantitative, a strain exhibiting high virulence for the guinea pig was tested in self-experiment. In the search for such strains, the colony morphology markers introduced by SERÉNY [48] and by KERÉKES [49] were also utilized. Using these markers, the Pécs team [50] was the first to find a highly guinea pig virulent strain. A *S. flexneri* 2a strain, of which 500 germs caused keratoconjunctivitis, was also found in our laboratory. In a self-experiment in 1964 [47], intake of 2000 such germs caused mild symptoms, and superinfection with a dose of 200 000 before the development of serious symptoms led to enterocolitis with blood in the stools accompanied by a fever of 39°C.

For the isolation of virulence factors, enzymes, and toxins, a strain containing mostly virulent, (and toxic) cells should be needed. For the selection of such virulent bacteria, a separation of the various sections of the non-homogeneous colonies should be performed. Virulence should be studied directly without subculturing the bacteria isolated from the separated colony sections. The morphological markers observed within the colonies should be assessed for virulence according to SERÉNY [48] and KERÉKES [49], and according to more recent methods. If, owing to genetic reasons, isolation and subculturing fail to produce such virulent cultures (as in the case of *S. sonnei*), physical methods, e.g. the separation of virulent and non-virulent bacteria by column chromatography, should be applied. The assumed pathogenic principle and viru-

Table XII
Susceptibility of the eyes of various animals

Guinea pig	++++	Ulcerated keratoconjunctivitis
Rabbit	++++	Ulcerated keratoconjunctivitis
Golden hamster	+++	Ulcerated keratoconjunctivitis
Gopher	++	Ulcerated keratoconjunctivitis
Mouse	++	Ulcerated keratoconjunctivitis
Rat	++	Ulcerated keratoconjunctivitis
Monkey	—	
Monkey after polio infection with paralysis	+	Non-ulcerated keratoconjunctivitis
Goat	++++	Non-ulcerated keratoconjunctivitis
Horse	++++	Non-ulcerated keratoconjunctivitis
Cat	+	Conjunctivitis
Dog	—	
Pig	—	
Chicken	—	
Hen	—	

lence factors could be prepared and detected by qualitative chemical and biochemical reactions with a much higher probability from a population in which nearly every individual is virulent than from a strain where only one among a million individuals is virulent.

The observation of LEVINE *et al.* [51] that as few as 10 germs of their *S. shigae* strains are already infectious to man shows the unpredictable significance of such virulent strains.

In contrast to data in the literature suggesting that keratoconjunctivitis cannot be induced in the mouse, strains capable of doing so have been observed by us. The susceptibility of the eyes of various animals is shown in Table XII as estimated by us.

Do differences in susceptibility reflect those in the macro or in the micro-organism? Do cultures have a stable behaviour in this respect? These offer a possibility for the separation of further virulence factors.

Table XIII shows the pathogens studied by us with some suspected to cause an eye reaction [52]. A highly contagious shigelloid *E. coli* strain spreading among guinea pigs has also been observed by us — though the infection could hardly be transferred even with eye discharge.

A similar question has been raised by GANGAROSA *et al.* [53] in human relation. The increased spreading capacity of some strains may perhaps be explained by the high stability of almost all of their colonies (very high average virulence) both under natural and laboratory circumstances.

Self-experiments with Yersinia enterocolitica. In 1965, we thought to have found a new enteric pathogen. The strain induced severe conjunctivitis in the guinea pig. In man, a dose of 300 million germs and a subsequent dose of 3×10^9 used for superinfection two days later, caused acute febrile enterocolitis and a mild subacute enterocolitis lasting for 2–3 weeks [54]. The strain was later identified as *Y. enterocolitica*. According to data in the literature *Y. enterocolitica* fails to induce lesions in the guinea pig eye [55], but we have succeeded in proving the contrary [56, 57]. Human infection shows a definite winter seasonality, and causes high serological titres [58], infective foci in the lymph nodes, febrile enterocolitis as well as other symptoms. Based on these signs, we think that *Y. enterocolitica* is more invasive, and has a closer parenteral relation with the organism than shigellae, though our attempts to detect *Y. enterocolitica* from the organs of guinea pigs 3–72 hr after eye inoculation have been unsuccessful. Though the pathomechanism is still obscure, the positive eye reaction suggests a close correlation between *Y. enterocolitica* and the other pathogens causing a mucosal inflammation. Our epidemiological data indicate a definite seasonality around winter and also show that a respiratory onset is perhaps not uncommon with *Y. enterocolitica*.

The results of screenings show that 1–2 thousand cases of acute yersinia infection must be reckoned with in Hungary yearly. Is vaccination necessary?

Table XIII

Pathogens causing keratoconjunctivitis or conjunctivitis in guinea pig eye

Bacteria	
<i>Shigella</i>	Keratoconjunctivitis
<i>Escherichia coli</i> (shigelloid)	Keratoconjunctivitis
<i>Listeria monocytogenes</i>	Keratoconjunctivitis
<i>Yersinia pseudotuberculosis</i>	Keratoconjunctivitis
<i>Yersinia enterocolitica</i>	Conjunctivitis
<i>Salmonella</i>	Conjunctivitis
<i>Neisseria gonorrhoeae</i>	Keratoconjunctivitis (literary data)
<i>Corynebacterium diphtheriae</i>	Keratoconjunctivitis (literary data)
<i>Pseudomonas aeruginosa</i> (with injury)	Keratoconjunctivitis
<i>Yersinia pestis</i>	?
<i>Francisella tularensis</i>	?
Viruses	
Adenovirus	Keratoconjunctivitis
Herpesvirus	Keratoconjunctivitis
Poxvirus variolae	Keratoconjunctivitis

Table XIV

Organs possessing mucous or other membranes suitable for studying infection and epithelial phase

Eye	Joints
Respiratory tract	Placenta
Bowel	Chorioallantoic membrane, e.g. in chick embryo
Vagina	Cell culture
Uterus and adnexa	
Other parts of urogenital tract	Meninges? - Serosa?

Experiments with Listeria monocytogenes. Oral intake of 100×10^9 germs of eye negative *L. monocytogenes* strains resulted in a few days excretion without any symptoms. Consequently, there are strains which can be used for human experiments without danger. Guinea pig eye positive strains have not been experimented with partly in view of their dangerous nature and partly in the interest of our female collaborators.

May *L. monocytogenes* become an enteric pathogen? In our laboratory, *L. monocytogenes* strains have been isolated from soup associated with food

Table XV

*Infectious doses of enteric pathogens in human volunteers**

Group	Infectious dose		Note	Guinea pig eye reaction
	other authors	own		
<i>E. coli</i> (infantile enteritis)				
O111 live	$10^7 - 10^{10}$			
O55 live	$10^7 - 10^{10}$			
O127 live	$10^6 - 10^7$			
<i>E. coli</i> (shigelloid)				
O143 live		10^9	Blood in stools, fever of 39°C (Öskü, 1958)	+ 10^5
O124 live	5×10^5 (?)	10^4	Mild symptoms, subfebrility (1963)	+ 3×10^4
<i>S. flexneri</i>	$10^9, 10^{11}$			+ $10^5 - 10^7$
2a live	$10^4 - 10^5$	2×10^3	Mild symptoms	+ 5×10^2
2a live		2×10^5	Superinfection. Blood in stools 8–10 times a day. Fever of 39°C (1964)	+ 5×10^2
2a killed 65°C		10^{13}	Intractable vomiting, intoxication (1957)	+
1b		2×10^{13}	Gastroenteritis? Intoxication (1955)	—
1b killed 100°C		2×10^{13}	—	—
<i>S. sonnei</i> I	10^9			+ $10^5 - 10^7$
<i>S. sonnei</i> I		2×10^{13}		—
<i>S. sonnei</i> II		2×10^{13}		—
<i>Y. enterocolitica</i>		3×10^8	Mild beginning symptoms	+ Conjunctivitis
		2×10^9	Superinfection, prolonged febrile enterocolitis, fever of 38°C (1965)	+ Conjunctivitis
<i>L. monocytogenes</i>		1×10^{11}	— (1971)	—
Enterocolitic faecal filtrate		5 g faecal filtrate	Mild enterocolitis (1971)	
<i>Y. enterocolitica</i> (inhalation)		?	— (1973)	+ Conjunctivitis

* For *E. coli* 0111 see the Addendum

poisoning. In view of the positive eye reaction, it may be assumed that *L. monocytogenes* can be pathogenic also for other mucous membranes.

Experiments on human volunteers and vaccination. My self-experiments with oral *Shigella*, shigelloid *E. coli*, *Y. enterocolitica* and *L. monocytogenes* infections were started in 1955 and similar human experiments performed all over world have long proved that such trials do not carry too much of a hazard, especially in the possession of antibiotics (Table XIV). A few human volunteer experiments and own data are summarized in Table XV.

Though the behaviour of the mucous membranes in the different animal models often show a surprising but not predictable parallelism with that of some mucous membranes of man, e.g. the guinea pig eye with the human colon, the most adequate experimental subject is man himself. For this very reason, the rigid standpoint of considering experiments of human volunteers completely inadmissible, is not reasonable. In my opinion, immunization experiments in a number of healthy volunteers with *E. coli* O124, the strain which during the 1958 epidemic affected 30 000 persons and caused disease in 10 000 without any severe sequelae, would have been justified and permissible. Their positive result would have considerably promoted the matter of vaccination. And, if it is permissible to send man into space, man must have the right to take the incomparably lower risk of human vaccination experiments, but only on a voluntary basis.

Addendum. After submitting this paper for publication, in the summer of 1973 new-type serial self-experiments were made with a late lactose-fermenting, *E. coli* O111 strain originating from a boy suffering from febrile diarrhoea. Twenty-four hours after a starting dose of 9×10^5 , diarrhoea with 38.5°C temperature and 7 watery stools on the first day developed. Without treatment the symptoms ceased after 3 days. In four steps gradually increased doses up to 2.5×10^{10} were ingested within 2 weeks without symptoms. Two days later a dose of 6×10^{11} caused a mild intoxication without diarrhoea. The duodenal juice examined once after 2 1/2 months was positive. Excretion of the organism in faeces lasted for 3 months. Pathogenic and immunogenic properties (enterotoxin?) of the organism, causing a surprisingly rapidly developing very high degree of immunity of questionable nature are under investigation.

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ЖИВАЯ ДИЗЕНТЕРИЙНАЯ ВАКЦИНА ФЛЕКСНЕРА 2а И ЗОННЕ

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BELAYA, YU., A., GEKKER, V. D., KUZMIN, S. N., SERGEEVA, N. S.: **Live *Shigella flexneri* 2a and *Shigella sonnei* vaccines.** Spontaneous mutants *Shigella flexneri* 2a 516 m and *Shigella sonnei* 6S are unaltered in antigenic structure and are capable of giving rise to microscopic changes in the mucosa. As shown in guinea pig eye test and in monkeys they exert a definite immunogenicity. They were not harmful in $1-75 \times 10^9$ doses to human volunteers and in 300×10^9 doses to monkeys. Passage of the mutants in humans, monkeys, chick embryos and in the guinea pig's eye failed to increase their virulence and to produce virulent revertants. Weak reactivity has been proved by oral administration of the freeze-dried vaccine in special capsules to humans.

Резюме. Селекционированы стабильные вакцинные штаммы шигелл Флекснера 2а 516 м и Зонне 6S, представляющие собой спонтанные мутанты с неизменной антигенной структурой и резко ослабленной вирулентностью. Остаточная вирулентность штаммов выражается в способности вызывать быстропроходящую, определяемую лишь при микроскопическом исследовании воспалительную реакцию слизистой. Установлена иммуногенность штаммов при изучении в кератоконъюнктивальном тесте на глаза морских свинок и в опытах на обезьянах. Вакцинные штаммы не вызывают заболевания при введении добровольцам в дозах от 1 до 75 млрд и при введении обезьянам до 300 млрд живых микробных клеток. Пассирование вакцинных штаммов через организм человека, обезьян, куриные эмбрионы и глаза морских свинок не увеличивает их вирулентности и не приводит к возникновению вирулентных ревертантов. Живая дизентерийная вакцина может быть изготовлена в виде драже, покрытых специальной оболочкой, и в лиофилизированном виде. Установлена слабая реактогенность и безвредность вакцины для людей при энтеральном применении. Наряду с нарастанием антител и усилением фагоцитарной реакции одним из важных механизмов местного иммунитета при дизентерии является возникновение устойчивости эпителия слизистой к пенетрации вирулентных шигелл.

Дизентерия продолжает оставаться серьезной проблемой для многих стран мира. В последние годы разрабатываются живые дизентерийные вакцины для перорального применения. В качестве вакцинных штаммов используются различные аттенуированные штаммы — спонтанные мутанты [1—5], стрептомицинзависимые штаммы [6—11], гибридные культуры шигелл и эшерихий — [12—15].

Селекционированные нами [4] вакцинные штаммы Флекснера 2а 516 м и Зонне 6S получены в результате многократного пассирования на искусственных питательных средах и отбора с помощью метода микроскопирования колоний в коспадающем пучке света спонтанных авирулентных мутантов с неизменной антигенной структурой и высокой иммуногенностью.

Как показали наблюдения в течение 8 лет, вакцинные штаммы оказались стабильными в своих морфологических, культуральных, ферментативных свойствах.

Таблица I

Биологические свойства вакцинных и исходных вирулентных штаммов шигелл

Штаммы	Кер. кон. проба	Вирулент- ность на куриных эмбрионах (микро. кл.)	Нер-2		Мышьяные перитоне- альные макро- фагии	
			пенет- рирую- щая способ- ность 5 ч.	цитотоксическое действие 9 ч.	Фагоцитар- ный показатель 1 ч.	цитотоксическое действие 3 ч.
Флекснер 2a 516 м вакцинный	—	> 200	7*	0**	7,3+0,9***	16,8
Флекснера 2a 516 вирулентный	+	2—20	15	40	8,3+2,8	51,5
Зонне 6S вакцинный	—	> 200	3	0	1,6+0,63	4,0
Зонне № 478 вирулентный	+	2—20	10	30	2,2+0,72	40,2

Обозначения: * — % инфицированных клеток

** — % клеток, подвергшихся цитотоксическому действию

*** — среднее количество бактерий в макрофаге.

Вакцинные штаммы сохранили также резко ослабленную вирулентность (табл. 1.).

Они не вызывают макроскопически определяемой кератоконъюнктивальной инфекции на глазе морских свинок при введении в дозах, в 1000 раз превышающих минимальную инфицирующую дозу исходных вирулентных культур. Штаммы не вирулентны для развивающихся куриных эмбрионов, обладают более слабой, чем вирулентные бактерии, проникающей способностью в культуре клеток Нер-2, не размножаются при этом внутриклеточно и не вызывают цитотоксического действия. При изучении в культуре мышьяных перитонеальных макрофагов они также, как и вирулентные культуры, поглощаются макрофагами, однако, в отличие от последних обладают менее выраженным цитотоксическим действием.

Вместе с тем, вакцинные штаммы Флекснера 2a и Зонне имеют некоторую степень остаточной вирулентности, что обнаруживается при гистологическом изучении срезов тканей глаза морских свинок и слизистой кишечника обезьян макаков резусов после введения этим животным бактерий вакцинных штаммов [16, 17].

Основными характеристиками вакцинных штаммов являются стабильность признака ослабленной вирулентности, безвредность и иммуногенность.

Стабильность ослабленной вирулентности вакцинных штаммов и их неспособность ревертировать в вирулентное состояние были прослежены

в течение 8 лет путем многократных повторных испытаний их в кератиконъюнктивальной пробе. Наряду с этим было проведено 15-ти кратное последовательное пассирование вакцинных штаммов на глаза морских свинок и 10-ти кратное пассирование на развивающихся куриных эмбрионах, являющихся высокочувствительной моделью для вирулентных шигелл. В этих опытах не было выявлено повышения вирулентности вакцинных штаммов.

Для определения стабильности и безвредности вакцинных штаммов были проведены специальные опыты пассирования вакцинных штаммов на

Таблица II

*Пассажи вакцинных штаммов на людях.
Вакцинный штамм Флекснера 2а 516 м*

Пассаж	Добровольцы	Доза ($\times 10^8$ живых мкр. клеток)	Количество выделенных штаммов шигелл	Вирулентность в кер. кон. пробе
I ↓	Б.	25	12	—
II ↓	Т.	45	16	—
III ↓	С.	50	3	—
IV ↓	Ф.	50	16	—
Вакцинный штамм Зонне 6S				
I ↓	Ср.	12	24	—
II ↓	М.	10	30	—
III ↓	Р.	15	31	—
IV ↓	Мл.	17,5	3	—
Всего			135	—

добровольцах (табл. II). Взрослые лица получали от 10 до 50 млрд живых микробных клеток лиофилизированной вакцины Флекснера 2а 516 м или Зонне 6S отдельно. Ежедневно в течение 2-х недель проводилось клиническое наблюдение, термометрирование и бактериологическое исследование.

После выделения и идентификации вакцинных штаммов от одного добровольца культуры выращивали на мясопептонном агаре и давали в виде смеси следующему добровольцу. Было проведено таким образом 4-кратное пассирование каждого вакцинного штамма — Флекснера 2а 516 м и Зонне 6S. Все выделявшиеся культуры шигелл испытывались в кератоконъюнктивальной пробе.

Исследование показало (табл. 2), что пассирование вакцинных штаммов через организм людей не вызывало увеличения вирулентности вакцинных штаммов и не сопровождалось появлением каких-либо клинических проявлений заболевания. Вакцинные штаммы выделялись в течение

1—3, реже 8 суток после введения и были авирулентны. Из изученных 135 культур шигелл не было выделено ни одного вирулентного ревертанта.

Для определения максимально переносимой дозы группе добровольцев, состоявшей из 50 человек, был введен вакцинный штамм Зонне 6S в возрастающих дозах от 10 до 50 млрд живых микробных клеток. Дозы от 10 до 40 млрд не вызывали никаких реакций. У семи из 10-ти человек, получивших по 50 млрд бактерий вакцинного штамма, наблюдались слабые местные реакции (метеоризм, запор), температура не повышалась. Из кала этих лиц выделено 68 культур бактерий Зонне, которые были авирулентны при испытании в кератоконъюнктивальном тесте.

Аналогичные данные были получены при введении вакцинного штамма Флекснера 2а 516 м. Всего в наших исследованиях на протяжении нескольких лет при изучении стабильности, безвредности и реактогенности вакцин было выделено около 1000 культур бактерий Флекснера 2а и Зонне от 240 людей и 77 обезьян, которым вводился тот или иной вакцинный штамм. Все штаммы были авирулентны. Среди них не было выделено ни одного вирулентного ревертанта. Вакцинные штаммы в этих опытах вводились в дозах от 1 до 75 млрд живых микробных клеток для человека и от 20 до 300 млрд бактерий для обезьян макака резус.

Одним из важных вопросов при оценке безвредности живых вакцин является маркировка вакцинных штаммов. Как известно, выделяющиеся от больных дизентерией шигеллы Флекснера, как правило, не обладают способностью продуцировать колицины. Количество колициногенных культур этого вида невелико и составляет не более 4%. Для маркировки селекционированного нами вакцинного штамма Флекснера 2а 516 м ему была наведена двойная колициногенность E_1 и V. Метка стойко сохранялась при хранении в лаборатории на питательных средах в течение 8 лет (срок наблюдения) и при пассивировании на лабораторных животных, обезьянах и человеке.

Кроме того, вакцинные штаммы Флекснера 2а 516 м и Зонне 6S были детально изучены по 25 различным признакам, характеризующим их ферментативные свойства в отношении углеводов, спиртов, аминокислот, изучены их колициногенные и колициночувствительные свойства, а также чувствительность к антибиотикам [18].

Эти данные необходимы для дифференциации вирулентных культур, которые могут быть выделены от иммунизированных лиц, и установления их происхождения.

В настоящее время на основе селекционированных вакцинных штаммов приготовлены моновакцины Флекснера 2а и Зонне в двух формах — в лиофилизированном виде в ампулах и в виде драже, покрытых специальной оболочкой, предохраняющей живые микробы от действия желудочного сока. Оболочка позволяет вводить непосредственно в кишечник вакцину в относительно небольших точно дозированных количествах.

Вакцина может храниться при -20°C в течение 2—3 лет, при $+4=+6^{\circ}\text{C}$ в течение 6 мес. — 1 года.

В опытах на 177 добровольцах установлена слабая реактогенность и безвредность вакцины Флекснера и Зонне в виде драже [19]. Определены

Таблица III

Реактогенность живой дизентерийной вакцины Флекснера и Зонне на добровольцах

Число лиц	Количество драже вакцины		Наблюдаемые реакции		
	Флекс- нера 2a	Зонне	интоксикация*	температура до $37,4^{\circ}\text{C}$	Местные реакции**
5	1	—	нет	нет	нет
5	—	1	нет	нет	нет
10	2	—	нет	нет	нет
10	—	2	нет	нет	нет
3	3	—	2	2	2
3	—	3	2	2	1
80	1	1	3(3,8%)	3(3,8%)	2(2,5%)
31	2	2	6(19%)	4(12,9%)	8(25,8%)

* — чувство недомогания, тяжести в голове, ускорение пульса

** — ощущение дискомфорта в животе, неприятные ощущения в области живота, метеоризм, запоры.

переносимые дозы вакцины и схема иммунизации. Введение вакцины в установленных дозах вызывало слабые местные и общие реакции у 3—5% лиц (табл. III).

В крови лиц, иммунизированных вакциной Флекснера, отмечалось нарастание агглютининов, при введении же вакцины Зонне сывороточные антитела не обнаруживались.

В настоящее время проводится работа по выявлению копроантител и определению роли их в постинфекционном и поствакцинальном местном иммунитете при дизентерии.

При экспериментальном изучении механизмов местного иммунитета, возникающего после введения живой дизентерийной вакцины, установлено (табл. 4), что местная иммунизация способствует резкому уменьшению количества и степени инфицирования эпителиальных клеток слизистой при последующем заражении вирулентной культурой. Так, у неиммунизированных контрольных животных при заражении вирулентной культурой инфицируется 25—56% эпителиальных клеток у всех 100% зараженных животных; в группе вакцинированных животных заражение вирулентной

Таблица IV

Биологическая и цитологическая характеристика местного иммунитета на модели дизентерийного кератоконъюнктивита

№№ морских свинок	Группы свинок	Через 2 суток после заражения вирулентной культурой Флекснера 2а			
		Кер. кон. проба	Количество микробов в отделяемом глаза	Количество инфицир. эпит. клеток в отпечатках	Интенсивность размножения диз. бакт. в эпит. клетках
1	I иммунные	—	0	0	0
2		—	0	0	0
3		—	3	0	0
4		—	1	0	0
5		—	0	0	0
6		—	0	0	0
7		—	0	5	2
8		—	0	0	0
9		—	4	0	0
10		—	0	4	31
11		—	1	5	3
12		—	3	0	0
13		—	0	7	4
14		—	0	0	0
15		—	39	3	3
16	II контроль	+	140	50	12
17		+	210	35	15
18		+	137	38	20
19		+	200	31	31
20		+	160	43	25
21		+	88	38	18
22		+	190	45	15
23		+	160	36	11
24		+	100	56	21
25		+	63	41	21

культурой приводит к инфицированию лишь 3—7% клеток, у 33% животных. Инфекционный процесс при этом не развивается.

Таким образом, в результате местной иммунизации живой дизентерийной вакциной возникает защитный механизм, направленный против пене-трации вирулентных шигелл в эпителиальные клетки слизистой, и таким образом препятствующий возникновению инфекции на самом первом его этапе — проникновении вирулентных шигелл в слизистую оболочку.

Установлено также, что в процессе дизентерийной инфекции и иммунизации происходит усиление фагоцитарной реакции макрофагов.

В заключение необходимо отметить, что в последние годы имеются существенные сдвиги в теоретическом изучении специфической профилактики дизентерии.

Однако необходимо констатировать, что для получения серьезных успехов в интересующей нас проблеме необходимо не только расширить исследования в отношении испытания эффективности предлагаемых живых дизентерийных энтеральных вакцин, но также продолжить изучение биологии возбудителей, патогенеза и механизмов иммунитета при дизентерии, а также, что очень важно, факторов естественного иммунитета организма, ибо только на основе этих знаний можно добиться получения эффективных методов профилактики дизентерийной инфекции.

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IMMUNITY AGAINST DYSENTERY AFTER ENTERAL IMMUNIZATION WITH LIVE VACCINE

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Summary. Experimental studies of immunity against dysentery were performed on rabbits after enteral immunization with live *Shigella flexneri* 2a vaccine. Immunization was carried out by a single injection of 40, 80, 120 or 150×10^9 bacterial cell live vaccine prepared from a streptomycin-dependent strain. By means of an artificially created fistula in the small intestine, intestinal juice was obtained at different periods after immunization and was examined for antibody content. As a result of the enteral immunization, a local and systemic humoral immune response of the primary and secondary type was obtained. Its degree depended on the dose of the vaccine and the interval between the first and the second antigenic stimulus. The maximum response manifested itself between the 7th and 14th days, persisting at a lower level after the 21st day. The local immunity caused an increased resistance of the intestinal mucosa against the virulent *S. flexneri* 2a strain, tested by means of the ileal loop method.

Studies of the epidemiological efficiency of immunization against dysentery with live vaccines is already in the field-trial stage. The data collected speak in favour of immunization with live vaccines prepared from streptomycin-dependent or attenuated strains, which induce a 3 to 12 months type-specific immunity [1-3]. In studies along the same lines, ISTRATI and MEITERT pointed not only to the induction of type-immunity, but also to a non-specific protection against other dysentery causing agents elicited by attenuated strains [4-6]. These data give support to the opinion that the immunogenic role of common humoral and cell mechanisms as well as of antibodies synthesized locally in the intestines has not been elucidated. In this situation, additional studies were needed to clarify the mechanism of immunity to dysentery in connection with the application of live vaccines. Therefore, an investigation was made into some aspects of the humoral and local immune response in immunization with live dysentery vaccines.

A *Shigella flexneri* 2a streptomycin-dependent strain was employed as vaccine. As test animals rabbits of the chinchilla and New Zealand breed, weighing 2.5 kg each were used. After laparotomy a cannula was introduced in the upper part of the small intestine. The fistula allowed to obtain and study the intestinal contents for the presence of antibodies and to administer the vaccine directly into the bowel for enteral immunization.

Basic immunization was performed by injecting doses of 40, 80 or 120×10^9 cells of live vaccine. A second dose of 120×10^9 cells of the same vaccine

was given after 22 days. In a further scheme, 150×10^9 cells were administered enterally, and the same dose was fed orally 14 days later.

(1) Prior to and on the 7th, 10th, 14th, 21st and 28th days after immunization blood samples were obtained and the agglutinin content was examined by the usual technique, while the passive haemagglutination test (PHT) was employed for estimation of the haemagglutinin content.

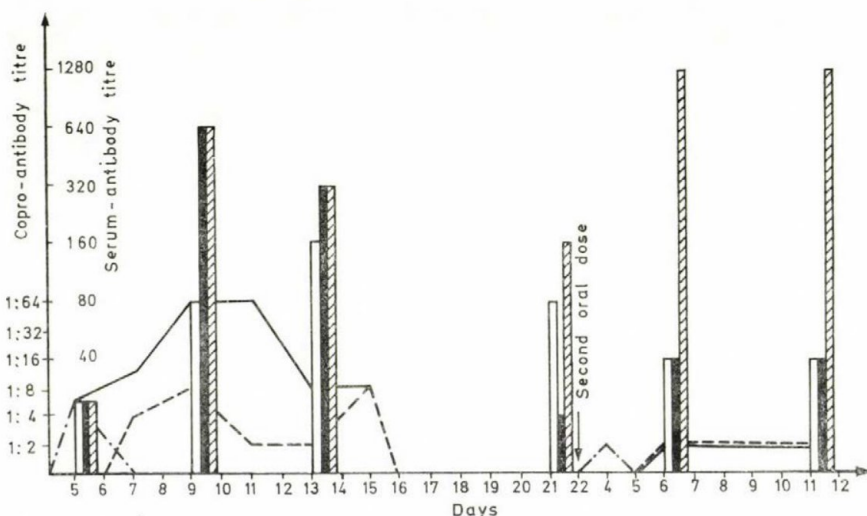


Fig. 1. First and second immune response after immunization with live dysentery vaccine (passive haemagglutination). Empty columns: Group I 20×10^9 ; solid columns: Group II 20×10^9 ; shaded columns: Group III 40×10^9 .

(2) From the 5th to the 23rd day after immunization and re-immunization, the faeces and the bowel content were examined daily for the presence of antibodies by means of agglutination and PHT.

(3) During 10 days after each vaccine dose, bacteriological inoculations of the faeces were carried out to isolate the vaccinal strain.

At different times after immunization, and on the 30th and 60th days, experiments were carried out to trace the degree of immunity by means of ileal loops in the immunized rabbits inoculated with increasing doses of live virulent *S. flexneri* 2a culture, such as 1×10^7 , 5×10^7 , 1×10^8 , 5×10^8 and 1×10^9 cells in 1 ml volume, the length of the loops being approximately 15 cm. The result was read after 24 hr, the presence and the quantity of secretion in the loops was determined and its contents was studied bacteriologically.

The presence of IgA-S in the bowel secretion was determined immunochemically and by immunoelectrophoresis.

It is seen from Fig. 1 that after the first antigenic stimulus, the strongest immune response was obtained with 120×10^9 germs as shown by the formation

of copro-antibodies in the course of two weeks. The second dose of 80×10^9 derms was followed by a considerably weaker immune response. The third dose of 40×10^9 germs produced only a slight increase of the haemagglutinins in intestinal contents on the 5th day. At the same time the haemagglutinin titres in the blood as determined by PHT were higher on the 9th and 13th days in the rabbits immunized with the smallest dose, being 1 : 80 in Group I against 1 : 640 in Groups II and III on the 9th day, and 1 : 160 against 1 : 320 on the 14th day. On the 21st day the titres in the three groups were 1 : 80 in the first, 1 : 20 in the second, and 1 : 160 in the third group.

Oral reimmunization carried out with a 120×10^9 dose caused a weak local immune response in the rabbits of Groups II and III. Antibodies were found on the 4th and 5th days only in Group III, while in Group II they displayed low values (1 : 2) from the 6th to the 11th day. This persisted in Groups I and II, while in Group III there was a statistically significant rise from 1 : 160 before reimmunization to 1 : 1280 on the 6th and 11th days after it. The titre on the 11th day after reimmunization was twice as high as that established on the 10th day after the first enterally administered dose.

Excretion of the vaccinal strain in the faeces continued for 3 to 4 days. In a single rabbit from the group immunized with 150×10^9 germs, excretion of the vaccinal strain was found to continue up to the 10th day after immunization; the isolated bacteria corresponded biochemically and serologically to the vaccinal strain.

The preliminary results of the ileal loop test seemed to indicate that in this manner the local immunity of the bowels could be challenged: they increased 2 to 10 times as compared those of the control animals.

Immunochemical investigations were also carried out to establish the quantity of IgA-S in the bowel contents. It was found to increase and persist until the end of the third week after the first dose.

A group of rabbits immunized with 150×10^9 cells gave a copro-antibody titre of 1 : 40 on the 7th day after the first dose, when the serum haemagglutinin titre was 1 : 320. The agglutinin titre rose from 1 : 40 before the immunization to 1 : 320 on the 7th and to 1 : 1280 on the 14th day. After oral reimmunization an increase of the range from 1 : 20 to 1 : 80 was observed from the 7th to the 23rd day in part of the animals. The rest failed to react to the reimmunization by producing copro-antibodies. At the same time, the serum haemagglutinin titre reached 1 : 320 on the 7th day and 1 : 160 on the 14th day, while the agglutinin titre rose from 1 : 1280 on the 5th day to 1 : 5120 on the 10th day, then dropped to 1 : 640 on the 14th, and to 1 : 320 on the 21st day.

All these data show that enteral immunization combined with oral reimmunization caused an immunologic change manifesting with a local synthesis of antibodies in the gut and in a general humoral reaction with a

significant increase of specific antibody content. Intestinal synthesis varied in dependence on the dose but continued only for 2 to 3 weeks. In all probability this was due to the short, 2 or 3 week interval between the first and the second antigenic stimulus, and to the increased IgA content in the intestinal juice.

On the basis of the results the following may be concluded.

(1) On enteral immunization with live dysentery vaccine prepared from a streptomycin-dependent strain, a local and systemic humoral immune response of the primary and secondary type was seen to develop.

(2) The degree of the immune response depended on the dose and the interval between the first and the second antigenic stimulus. A strong immune response was obtained with a dose of 1×10^8 or more bacteria.

(3) Development of the local and humoral antibody response was characteristic, with a maximum between the 7th and 14th days and its persistence at a lower level up to the 21st day.

(4) The immunity obtained has been found to cause an increased resistance of the intestinal mucosa against infection with the live virulent *S. flexneri* 2a strain, as shown by the results of the ileal loop method.

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SUCCESSFUL FIELD TRIALS WITH ORAL KILLED *SHIGELLA SONNEI* VACCINE

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Summary. Large-scale oral immunization with *Shigella sonnei* bacteria decomposed by freezing and thawing resulted in a significant decrease of the incidence of *S. sonnei* dysentery among vaccinated children as compared to the control groups.

Prophylaxis of *Shigella sonnei* infections represents for the most part a hygienic problem. The disease mainly affects infants as, owing to their insufficient personal hygiene, faecal-oral infections cannot be avoided. In infants' collectives there is a high risk of a rapid spread of dysentery, so that hygienic measures by themselves have no sufficient prophylactic effect.

In infants' homes and day-nurseries, excretors admitted cause a number of contact cases within the collective and these contact cases on their part become the infectious focus in families. Thus, collectives of children of preschool age are a real epidemiological turn-table of dysentery. Effective immunization of all children in the collectives would cause a considerable decrease in overall morbidity, as the frequency of *S. sonnei* infections in kindergartens and day-nurseries in the GDR amount to 60% of the total morbidity, although the number of children in these establishments amounts only to 5% of the total population (Fig. 1).

Parenteral immunization for dysentery is, however, unsuitable for children of 1—6 years of age, because the reactogenicity of injected vaccines is high, especially if frequent reimmunizations are needed. The danger of a so-called negative phase on the occasion of immunizations in the latency period also speaks against parenteral immunization, oral immunization on the other hand is suitable for use on a large scale without danger for the children.

The vaccine "Orovaccin-E-Ruhr" produced in the GDR for oral immunization against *S. sonnei*, in the form of oralets for children and adults, and in a granulated form for infants younger than 3 years, contains total antigens decomposed by freezing and melting. One oralet contains 50 mg of antigen. The vaccine is given in jam or fruit juice on an empty stomach 1—2 hr after a meal. The vaccine is tolerated well and it does not contain live shigellae or any other pathogenic or conditionally pathogenic microorganism.

In Department Halle the frequency of dysentery has been above average for years. Here in the Bitterfeld district was carried out the first epidemiological test in a field-trial in 1966. The district has 140 000 inhabitants and their dysentery morbidity has been higher for many years than in Department Halle. Six thousand children in establishments and their personnel of 1200 subjects were included into the trial. From November, 1966 to April, 1968, two campaigns

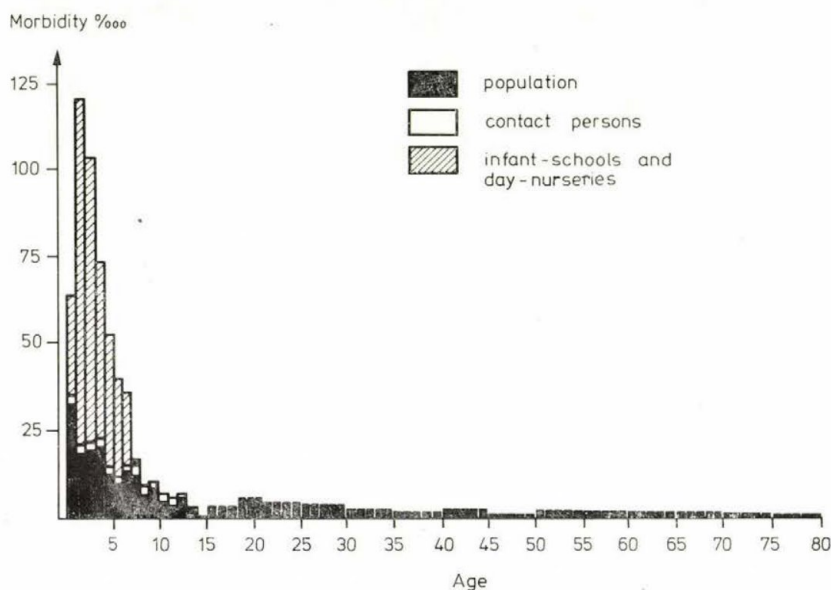


Fig. 1. Age distribution of cases of *S. sonnei* dysentery

were carried out in a double blind trial: 2995 children were immunized and 2992 children received placebo. The result was a lower frequency of illness among the immunized and the difference between these and the placebo group was significant.

From May, 1968 to August, 1970, this trial was followed by a second one, by vaccinating all children and persons working in establishments. Immunization was carried out in 4 parts within a year. On the occasion of each part, vaccination lasted 10 days by giving 500 mg antigen, i.e. 2000 mg a year. Babies were not immunized. During the 2 years of observation, a significant decrease of morbidity was observed.

In the institutions for children, during the 2 years before vaccination, 190 cases of dysentery were recorded, while in the 2-year period of vaccination only 6 cases, a decrease of 96.1%. During the 2 years before immunization, the

number of carriers amounted to 136, while in the 2 years after immunization to 6 (Fig. 2).

This result cannot be ascribed to the low number of examinations because every person admitted to children's institutions or homes for the aged and taking up work in the food industry, etc. are subjected to faecal examination. In this way, cases of diarrhoea, *Escherichia coli*, salmonella or dysentery

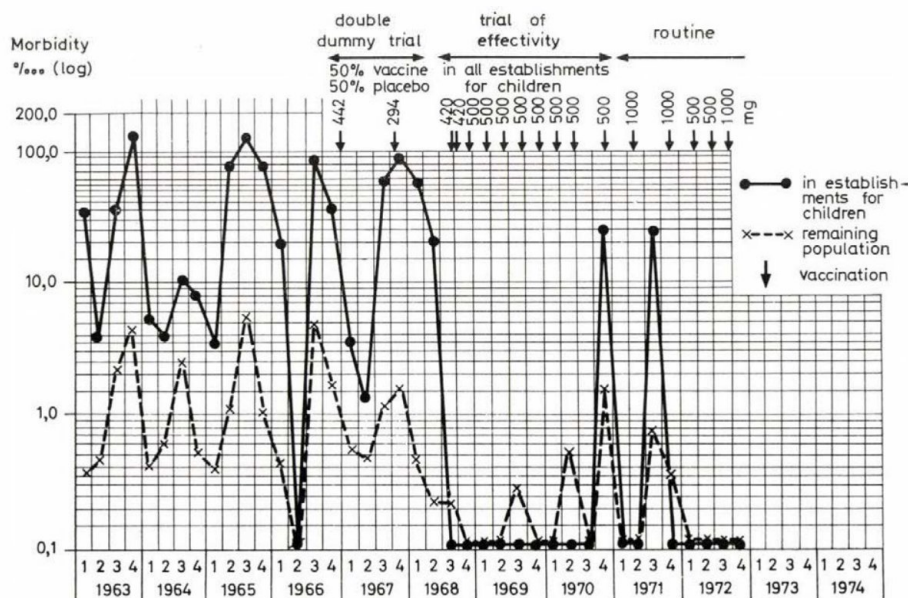


Fig. 2. Morbidity of *S. sonnei* dysentery in children's institutions and among the remaining population in the Bitterfeld district. The first peak (4th quarter of 1970) shows an accumulation of cases of illness in an infants' home at the end of the 4th month after vaccination, i.e. the revaccination was carried out too late. The second peak (3rd quarter of 1971) shows the cases of illness in a day-nursery. Children in day-nurseries were given no vaccine during this campaign

infections would have certainly be revealed. Taking this into consideration, a subclinical illness could have hardly caused an expansion of dysentery among the immunized population.

A comparison with other territories showed that the decrease in morbidity was not accidental. As a general epidemiological tendency, a decrease in morbidity could be noticed. In the Halle district, the decrease was, however, less than in the Bitterfeld district: the morbidity in the Halle district decreased without immunization by 52%, while in Bitterfeld by 93%. The difference was highly significant (Table I, Fig. 3).

Since autumn 1970, in the kindergartens of the Halle district about 105 000 children were vaccinated against *S. sonnei*.

Table I

Incidence of S. sonnei dysentery in the Bitterfeld District during a 2-year observation period

	Incidence of <i>S. sonnei</i> dysentery, No. of cases			
	before vaccination May 1, 1966 to April 30, 1968		after vaccination May 1, 1968 to April 30, 1970	
District Bitterfeld	358	(100.0%)	25	(7.0%)
Department Halle (without Bitterfeld)	3450	(100.0%)	1657	(48.0%)

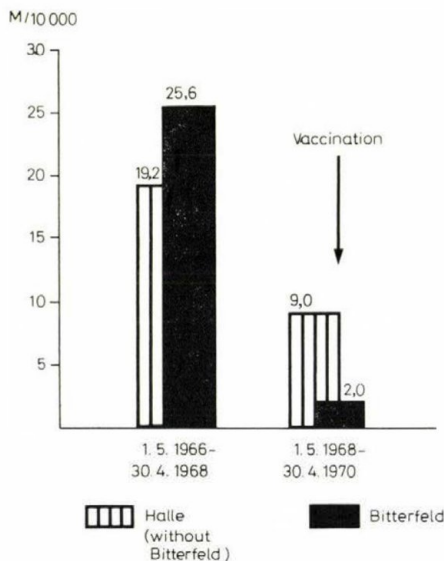


Fig. 3. Morbidity of *S. sonnei* dysentery

Experience showed that after a dose of 2000 mg protection persisted for 4–6 months. Thus, 3 campaigns seemed to be necessary and therefore in September 1000 mg antigen and in February and June 500 mg each were administered. The double dose in September was justified by the admission to the kindergartens of new age-groups.

In view of the higher incidence of the disease in kindergartens, the 1 to 3 year age-groups received 3×1000 mg antigen. After one year of observation in Halle district, in 1971 the incidence of *S. sonnei* infections was lowest since 8 years. A comparison of the morbidity of Halle with that of the 5 neighbouring districts and the whole of the GDR showed that during the 7 years before immunization, the Halle district had the highest morbidity rate of *S. sonnei* infections (Table II).

Table II

Morbidity of S. sonnei dysentery in Department Halle and in the neighbouring departments

Department	1972		1971		1970		1969		1968		1967		1966		1965		1964	
	Place	Morbidity	Place	Morbidity	Place	Morbidity	Place	Morbidity	Place	Morbidity	Place	Morbidity	Place	Morbidity	Place	Morbidity	Place	Morbidity
Halle	2	4.2	1	3.8	4	4.7	4	4.7	4	6.1	5	9.4	5	8.5	6	11.2	5	29.8
Potsdam	3	5.0	2	5.0	1	0.4	3	4.7	3	4.4	3	7.1	3	3.5	3	6.6	4	14.1
Leipzig	1	3.2	3	6.5	3	1.3	6	8.3	6	8.8	6	10.1	2	2.7	1	2.4	1	2.8
Magdeburg	5	8.4	4	8.5	2	0.8	5	5.7	5	6.2	4	9.2	4	4.7	5	9.1	6	32.5
Gera	4	5.3	5	10.4	5	7.1	1	2.7	1	1.5	1	3.4	1	2.3	2	2.9	2	10.0
Erfurt	6	18.8	6	11.6	6	13.7	2	4.1	2	2.0	2	4.0	6	10.4	4	8.6	3	10.4
G.D.R.		5.1		5.1		3.3		4.9		4.2		5.8		7.1		5.6		16.8

Evaluation of the incidence of *S. sonnei* infections among the vaccinated and non-vaccinated children in the immunized collectives showed that of the immunized 11.9%, while of the non-vaccinated 22% fell ill with dysentery. The use of oral immunization during epidemics was successful, because accumulations of contact cases could be avoided in a high degree. Earlier, these contact cases appeared especially after cases of illness in children's institutions and other collectives.

After massive alimentary infections, the course of illness is mitigated, but not wholly prevented, by immunization against *S. sonnei*.

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ORAL IMMUNIZATION AGAINST DYSENTERY

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Summary. The basic problems of artificial immunity against dysentery are its short duration and low intensity, necessitating frequent revaccinations. The only solution of the problem is to give the antigen orally. Experiments were carried out in mice and human volunteers vaccinated orally with killed or with different avirulent live bacteria. Efficient doses of these vaccines produce a high level of protective serum and faecal antibodies and this level can be maintained by weekly revaccinations. The protective antibody level ensured by killed vaccines can only be surpassed by live strains which have retained the intactness of their O antigen and tissue-penetrating capacity. Penetrating capacity, being associated with a limited proliferating activity in the tissues, is connected with undesirable toxicity. Furthermore, this type of vaccines presents problems during preparation, control, and maintenance, and there is always a possibility of virulence reversion. In our opinion, killed bacteria or a well-absorbed extract antigen of the Boivin-type should be given preference in view of its being safer, well standardizable, easier to administer and keeping its potency for long periods.

Artificial anti-dysentery immunity presents two basic problems, (i) the low intensity of immunity; (ii) the short, 3–4 months, period of protection.

In view of the short duration of immunity, repeated revaccinations are needed to prolong the period of protection. Problems of side reactions and other difficulties encountered with parenteral immunization have made us to study the ways of solving these problems by oral anti-dysentery vaccination.

In mice, a result equivalent to that of parenteral immunization was attained by administration of approximately 100-fold doses. Examination of antigens prepared in different manners indicated that higher amounts of antigen were required not because of the special situation or the structure of the intestinal RES, but simply owing to the different absorption rate. The 1 : 100 ratio of the subcutaneous : oral doses was found necessary and this ratio for an equivalent immune response is characteristic of corpuscular vaccines such as killed bacteria. This ratio decreases to 1 : 30 when a colloidal antigen, Boivin extract, is used for immunization. In these animal experiments the presence of protective serum and copro-antibodies has also been demonstrated. Furthermore, by weekly oral revaccinations with small amounts of antigen, immunity was maintained at a constant level [1, 2] (Table I).

Observations were collected also on human volunteers of different age groups. The vaccine was produced by freeze-dried *Shigella* antigens prepared from Boivin extracts which were precipitated with ethanol and compressed

Table I

Relative potency (RP) values for serum- and copro-antibodies in mice after primary and booster oral doses of Boivin antigen

Time	RP values of antibodies in animals			
	not revaccinated		revaccinated*	
	serum	intestinal	serum	intestinal
0 day	1	1	1	1
3 days	5	55	7	33
17 days	17	5	20	55
42 days	1	1	22	33

* Revaccination by 1/5th of dose weekly, after one week of priming

into tablets containing about 0.5 mg dry antigen. Primary immunization was carried out by 5 tablets as a single dose or by 3 tablets given twice in an interval of a week. After this primary immunization, a booster dose of one tablet was given weekly [3, 4].

This oral basic immunization ensured the same mouse-protective titre of the serum samples as that obtained in the subcutaneously vaccinated control group. The antibody level could be maintained for an observation period of 8 months by weekly oral revaccinations. In contrast, in the non-revaccinated group, immunity decreased rapidly (Table II).

FORMAL *et al.* [5], MEL *et al.* [6], as well as ISTRATI [7] and others studied oral anti-dysentery vaccination by the use of attenuated live strains. DUPONT *et al.* investigated different avirulent mutants and a hybrid strain in animal experiments and in human volunteers [8, 9]. MEL *et al.* tested a streptomycin-

Table II

Average relative mouse-protective potency against S. flexneri 2a, 3 and S. sonnei of sera from human volunteers immunized orally by Boivin-type antigen tablets

Time	RP values in groups	
	not revaccinated	revaccinated*
before vaccination	1	1
1 week after priming (2×3 tablets)	10.8	19.3
5 weeks after priming	2.6	14.7
10 weeks after priming	1	17
32 weeks after priming	1	12.3

* One tablet weekly

dependent strain [10], and ISTRATI worked with empirically selected avirulent strains in human volunteers.

Our experiments [11] were begun by selecting and characterizing different avirulent mutants of freshly isolated *S. flexneri* strains. Although we accepted the differentiation of avirulent mutants recommended by LABREC *et al.* [12], we have reached this point by using other methods. A group of frequently isolated avirulent mutants had lost its penetrating capacity as well as its multiplying capacity in the tissues. Such strains were negative in Serény's test, avirulent in 10-day-old chick embryos inoculated intravenously or into the allantoic cavity, and failed to cause mouse-carriership (Table III).

Table III
Classification of avirulent mutants on the basis of virulence tests

Test	Avirulent mutant classes		
	PC-MCT ⁻	PC-MCT ⁺	PC+MCT ⁻
Keratoconjunctivitis	negative	negative, $\pm$	negative
Mouse infectivity	negative	low	negative
Chick embryo intravenously	10 ³ germs	10 ¹ germs	10 ¹ germs
Chick embryo intraallantoic	negative	negative	10 ³ germs

PC = penetration capacity

MCT = multiplying capacity in tissues

The second class of avirulent mutants lost only the penetrating factor of virulence. Such clones showed very weak or no Serény-positivity, a low mouse infectivity, and were negative in the chick embryo allantois test: inoculated intravenously into chick embryos, these strains behaved as did virulent ones (Table III).

The third mutant class, with retained penetrating character without a growing capacity in tissues was represented by a *Shigella* $\times$ *E. coli* hybrid strain produced by FALKOW *et al.* [13] (Table III).

The immunogenicity of these avirulent mutant classes was compared to that of killed bacteria and of colloidal (Boivin) antigen in mouse experiments [14] (Table IV).

The results showed a similar level of protective effectiveness in classes of completely avirulent, penetration defective strains and killed bacteria. A somewhat higher immunogenicity was observed with the hybrid strain and colloidal antigen. The difference between these latter antigens was not significant statistically.

Results concerning immunogenicity were as expected. A strain having no penetrating ability cannot produce more stimuli than do killed bacteria. The

Table IV

Immunogenicity of formalinized vaccine, Boivin-type antigen and of clones representing different virulent mutants

Vaccine	ED ₅₀ values (No. of germs)
Completely avirulent (PC-MCT ⁻)	1.0 × 10 ⁸
Non-penetrating avirulent (PC-MCT ⁺)	8.0 × 10 ⁷
Penetrating, non-multiplying (PC ⁺ MCT ⁻) hybrid (M22-18 × 16)	8.0 × 10 ⁶
Formalinized vaccine	6.0 × 10 ⁷
Boivin antigen	2.0 × 10 ⁷ *

* Cell counts equivalent to amount of Boivin antigen were standardized by haemagglutination inhibition test

hybrid strain with preserved penetrating but limited multiplying capacity in tissues (as assumed by FORMAL) proved considerably more effective. The question is whether this result meant a qualitative difference, in other words, whether the strain had a special protective antigen responsible for its effectiveness. The other possibility is that the higher immunizing dose was responsible for the better immunogenicity. The ED₅₀ value is determined by the initial bacterial count introduced into the animal, provided these bacteria are multiplying. The effective dose, the actual quantity of antigens, will differ from the introduced one and therefore the basis of evaluation is incorrect.

There are some other facts which have to be taken into consideration when choosing a vaccine. To prepare a Boivin extract, making tablets, the process of standardization and control is a convenient method. Such tablets may be stored for years, even at room temperature. Their administration is easy, there is no danger, or a contraindication, and any quantity may be given to infants or even patients suffering from different diseases.

On the other hand, the use of an attenuated strain for vaccination needs a rigorous and permanent control of the strain's virulence in view of the danger of reversion. Many problems arise with the standardization, control, and transport of fresh preparations. Even the limited multiplying capacity of the hybrid strain applied may cause mild symptoms [5, 9].

Taking into account all the above, we do not recommend the use of live avirulent strains as vaccines, especially as a comparable immunizing effect can be reached by applying colloidal antigens. Such a vaccine can be given in a dose sufficient for priming and then the level needed for protection can be maintained infinitely by revaccinations.

Epidemiological observations could only confirm this experimental conclusion. In two collectives, about 300 mentally retarded children were

immunized during two years. According to RUDNAI [15], the few cases of dysentery did not allow to draw convincing conclusions as to the vaccines' efficacy.

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СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ В ЛАБОРАТОРНЫХ ТЕСТАХ СПЕЦИФИЧЕСКОЙ БЕЗОПАСНОСТИ ВАКЦИННЫХ ШТАММОВ ДЛЯ ЖИВЫХ ДИЗЕНТЕРИЙНЫХ ВАКЦИН

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JIKIDZE, E. K., KAVTARADZE, K. N., ELKINA, S. I.: **Laboratory safety testing of live dysen-
tery vaccine strains.** The guinea pig eye test, mouse "lung model", oral infection of monkeys
and cell culture method were applied to examine *Shigella flexneri* 2a 516 m, *Shigella sonnei* 6S,
streptomycin dependent mutants of *S. flexneri* 2a 1605/3 and *S. sonnei* L recommended for
the preparation of live vaccine. The spontaneous mutants showed a higher "residual" virulence
than the other cultures. The minimum permissible residual virulence has been determined.

Резюме. Проведено сравнительное изучение специфической безопасности спон-
танных мутантов шигелл Флекснер 2а 516 М и шигелл Зонне 6S, а также стрептомицин-
зависимых штаммов шигелл Флекснер 2а 1605/3 и Зонне Z, предложенных для живых
дизентерийных вакцин. В качестве лабораторных тестов оценки специфической безо-
пасности были использованы кератоконъюнктивальная проба на морских свинках, легоч-
ная модель на мышах, скормливание обезьянам и заражение клеточных культур. Уста-
новлена более выраженная «остаточная вирулентность» спонтанных мутантов. Приняты
минимальные требования к допустимой «остаточной вирулентности» дизентерийных
вакцинных штаммов.

Проблема специфической профилактики дизентерии продолжает оста-
ваться актуальной. Подкожная вакцинация оказалась недостаточно эффек-
тивной. В связи с этим во многих странах мира проводятся исследования
по разработке живых дизентерийных вакцин (Венгрия, ГДР, Югославия
Румыния, США и др.) для энтеральной иммунизации. В 1965, 1968 и 1971
годах Д. М. Мел [1—4] опубликовал результаты изучения в Югославии на
ограниченных контингентах людей эпидемиологической эффективности и
специфической безопасности живых дизентерийных вакцин из стрептоми-
цин-зависимых штаммов шигелл. Полученные им данные свидетельствуют
о безопасности и удовлетворительной эффективности этих вакцин.

В Советском Союзе живые дизентерийные вакцины разрабатываются
в двух институтах — в ИЭМ им. Гамалеи доктором Ю. А. Белой и в Москов-
ском НИИВС им. Мечникова — доктором В. В. Сергеевым.

Перспектива использования живых дизентерийных вакцин вызывает необходимость разработки лабораторных методов оценки их специфической безопасности и иммуногенной активности, а также разработки минимальных требований к качеству препаратов.

В настоящем сообщении представляются результаты исследований, проведенных сотрудниками ГИСК им. Тарасевича совместно с ИЭМ им. Гамалеи, Московским НИИВС им. Мечникова и Институтом экспериментальной патологии и терапии АМН СССР. Было проведено изучение специфической безопасности вакцинных штаммов спонтанных мутантов шигелл Флекснера 2а 516 М и шигелл Зонне 6S, полученных в ИЭМ им. Гамалеи Ю. А. Белой [5], стрептомицин-зависимых штаммов шигелл Флекснер 2а 1605/3, полученного в Московском НИИВС им. Мечникова В. В. Сергеевым [6], и Зонне Z, полученного К. Линде в ГДР. Все перечисленные штаммы были переданы в ГИСК им. Тарасевича на апробацию.

В качестве лабораторных тестов для оценки специфической безопасности были использованы кератоконъюнктивальная проба на морских свинках, скармливание обезьянам, легочная модель на белых мышах и заражение клеточных культур.

При испытании в кератоконъюнктивальной пробе на морских свинках по методу Шерени (1955) [7] вирулентные штаммы шигелл Флекснер 2а и Зонне вызвали гнойный кератоконъюнктивит, проникали и размножались в клетках эпителия конъюнктивы и роговицы. Вакцинные штаммы не вызвали макроскопически видимых изменений.

При гистологическом исследовании были выявлены незначительные явления конъюнктивита без выраженных изменений в роговице (рис. 1). При этом шигеллы стрептомицин-зависимых штаммов редко проникали в клетки эпителия, тогда как шигеллы спонтанных мутантов в незначительном количестве постоянно обнаруживались в клетках эпителия (рис. 2). Этот факт, по нашему мнению, свидетельствует о более высокой степени остаточной вирулентности спонтанных мутантов.

Под «остаточной вирулентностью» вакцинных штаммов мы подразумеваем резко ограниченную способность шигелл проникать в клетки эпителия при отсутствии способности к размножению и вызывать ограниченную воспалительную реакцию, определяемую только гистологически.

Многократное пассирование вакцинных штаммов в кератоконъюнктивальной модели на морских свинках не повышало уровня их «остаточной вирулентности».

В 1957 году для изучения патогенных кишечных бактерий Войно-Ясенецкой [8] была предложена так называемая «легочная модель». На этой модели Войно—Ясенецкий [9] показал, что вирулентные шигеллы проникают в клетки эпителия дыхательных путей, размножаются в этих клетках и разрушают их. Авирулентные штаммы не размножались в аналогичных

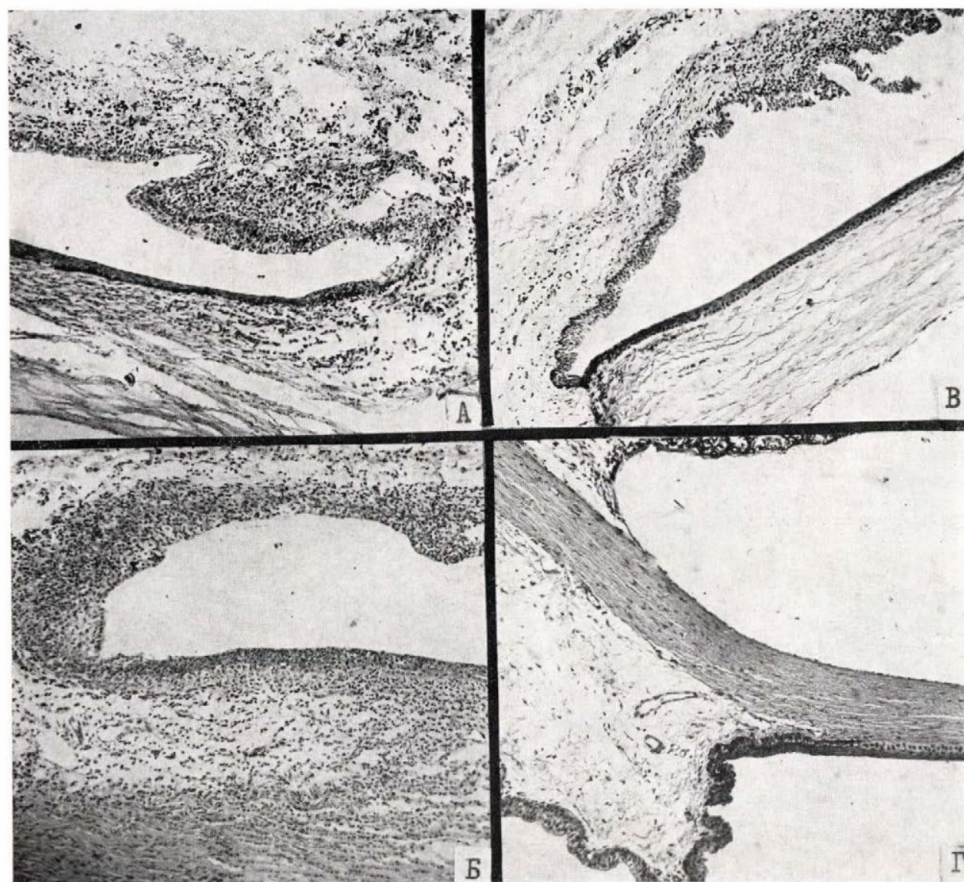


Рис. 1. Кератоконъюнктивальная проба на морских свинках. Окраска гематоксилин-эозином. Увеличение 106х. А. Вирулентный штамм Флекснера, 6 часов после заражения. Явления конъюнктивита с началом кератита. Б. Тот же штамм через 24 часа после заражения. Флегмонозноязвенный кератоконъюнктивит. В. Спонтанный мутант Флекснера, 6 часов после заражения. Незначительные явления конъюнктивита. Г. Тот же штамм, через 24 часа после заражения. Воспалительные изменения не прогрессировали.

условиях. Эти данные подтверждены в работах других исследователей [10—13]. «Легочная модель» была использована нами для изучения специфической безопасности вакцинных штаммов шигелл Флекснера и Зонне.

При испытании в «легочной модели» на мышах спонтанные мутанты шигелл Флекснер 2а 516 М и Зонне 6S, а также стрептомицин-зависимые штаммы шигелл Флекснер 2а 1605/3 и шигелл Зонне Z не вызывали гибели животных. В противоположность этому вирулентные штаммы шигелл Флекснер и Зонне вызывали гибель мышей, которая регистрировалась уже спустя 24 часа после введения культуры.

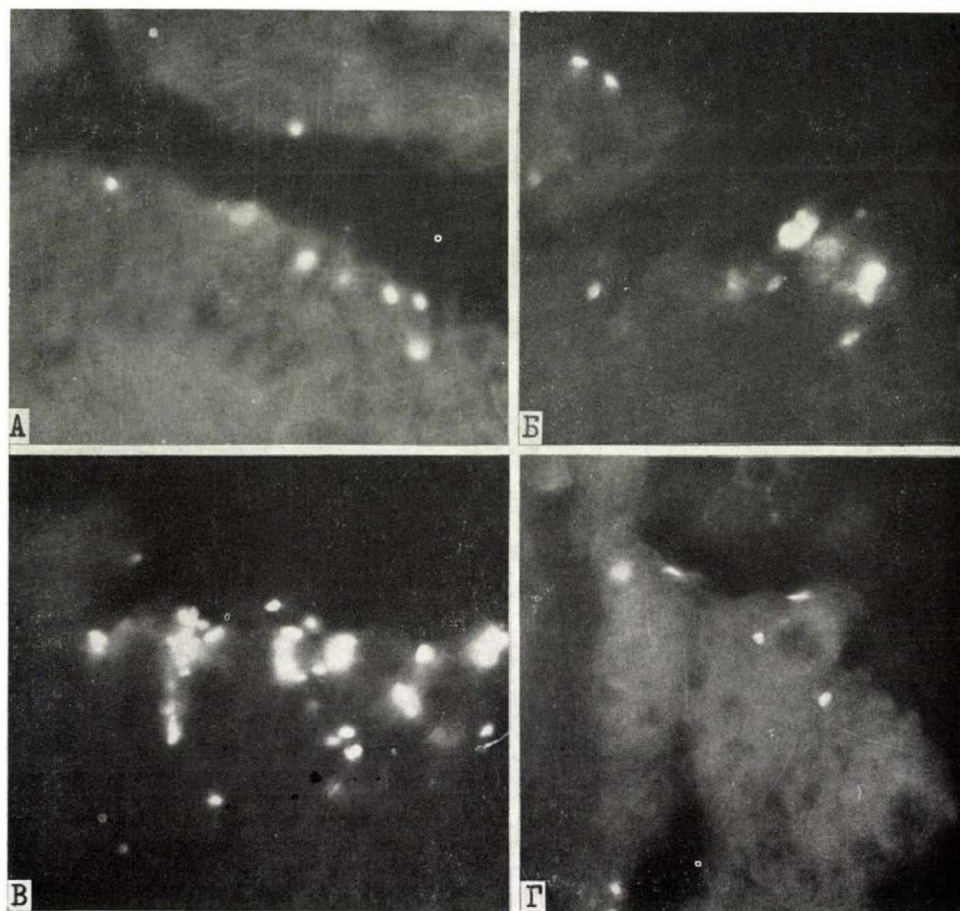


Рис. 2. То же. Люминесцентно-серологический метод. Увеличение 900х. А. Вирулентный штамм Флекснера 2а через 6 часов после заражения. Значительное число шигелл в эпителиальных клетках конъюнктивы. Б. Спонтанный мутант Флекснера 2а через 6 часов после заражения. Приблизительно такое же количество шигелл в эпителиальных клетках. В. Вирулентный штамм через 24 часа после заражения. Большое число шигелл в клетках эпителия конъюнктивы. Г. Спонтанный мутант через 24 часа после заражения. Единичные шигеллы в клетках эпителия конъюнктивы.

На графике (рис. 3) представлены сравнительные данные, полученные при изучении поведения в легочной модели на мышах вакцинных штаммов шигелл Флекснер, Зонне и вирулентных штаммов шигелл. Кривые показывают, что в отличие от вирулентных штаммов шигеллы вакцинных штаммов не накапливаются в легких, что свидетельствует об отсутствии их размножения. Следует отметить, что очищение легких мышей от шигелл стрептомицин-зависимых штаммов происходит быстрее, по сравнению с очищением животных от шигелл спонтанных мутантов. Эти данные совпадают с резуль-

татами кератоконъюнктивальной пробы, указывающими на то, что спонтанные мутанты обладают более выраженной остаточной вирулентностью.

В последние годы для изучения вирулентности патогенных бактерий, в том числе шигелл, широко используются культуры клеток. Выявлена прямая связь между уровнем вирулентности шигелл и их способностью размножаться в цитоплазме клеток [14—18]. Эти данные послужили основанием к использованию клеточных культур в качестве теста для оценки специфической безопасности вакцинных дизентерийных штаммов. Нами были

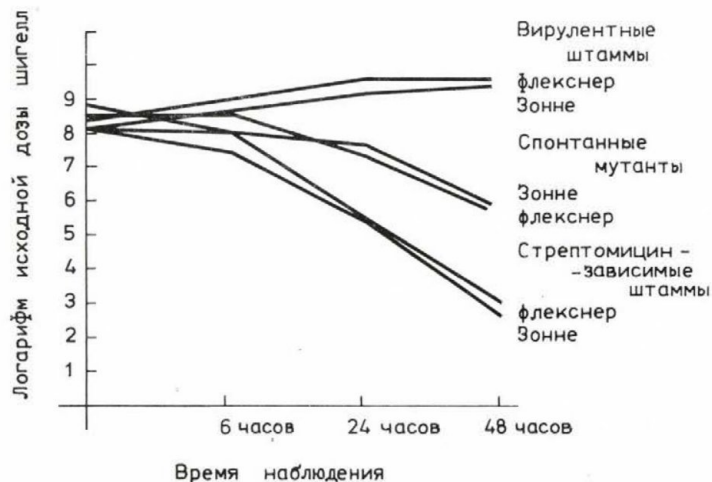


Рис. 3. График

использованы культуры клеток HeZa, Нер—2 и Fl. В течение всего периода наблюдения не было выявлено клеток с размножающимися в них шигеллами. В отличие от этого, при микроскопии препаратов клеточных культур, зараженных вирулентными штаммами шигелл Флекснер и Зонне, уже через 3 часа обнаруживали клетки, заселенные бактериями (рис. 4). Количество заселенных клеток возрастало к 7-му часу после заражения. К этому сроку бактерии целиком заполняли цитоплазму пораженных клеток. Таким образом, было установлено, что шигеллы вакцинных штаммов не обладают способностью размножаться в клетках культур *ин vitro*.

Для подтверждения специфической безопасности вакцинных штаммов шигелл Флекснер и Зонне представлялось целесообразным изучение их в опыте на обезьянах Макака резус, чувствительность которых к дизентерии и течение этой инфекции имеют много общего с дизентерией у человека [19—22]. При испытании на обезьянах оказалось, что многократное скормливание вакцинных штаммов (общая доза 200—250 млрд. микробных тел) не вызывало клинических проявлений дизентерии у животных. В то же время скорм-

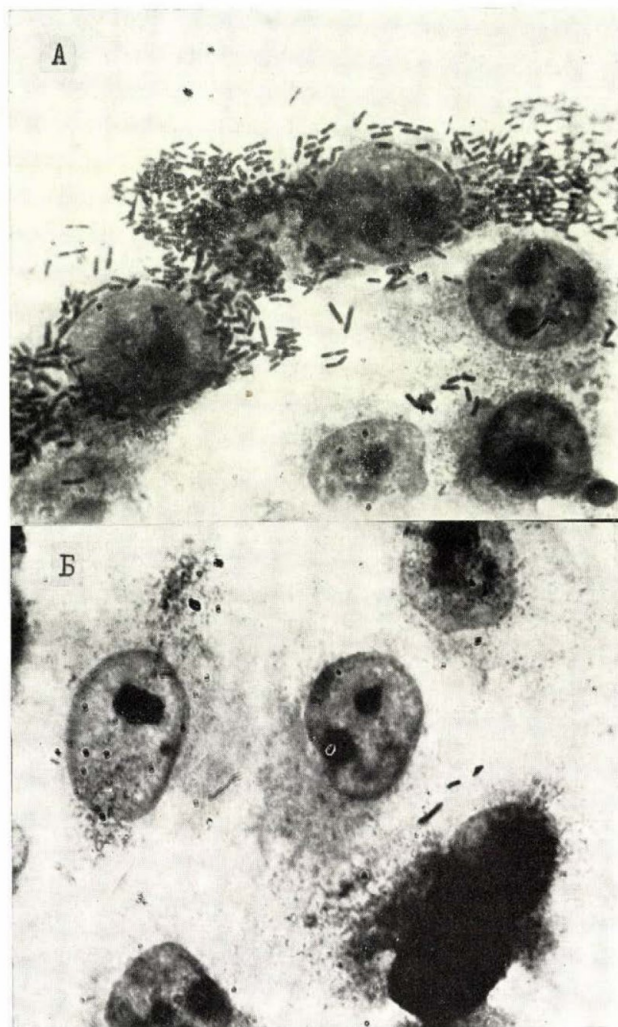


Рис. 4. Вирулентный и вакцинный штаммы шигелл в культуре клеток Нер—2. Окраска гематоксилин-эозином. Увеличение 1000х. А. Размножение шигелл вирулентного штамма Флекснер 2а в клетках. Б. Культура клеток, зараженная стрептомицин-зависимым штаммом шигелл Флекснера 2а 1605/3.

ливание вирулентных штаммов приводило к заболеванию со всеми характерными симптомами дизентерии. При гистологическом исследовании органов животных, получивших вакцинные штаммы, были обнаружены лишь незначительные очаги воспаления в слизистой оболочке кишечника (рис. 5). Необходимо отметить, что шигеллы стрептомицин-зависимых штаммов редко проникали в клетки эпителия слизистой кишечника (рис. 6). При скормли-
вании спонтанных мутантов шигелл Флекснер и Зонне, последние в незна-

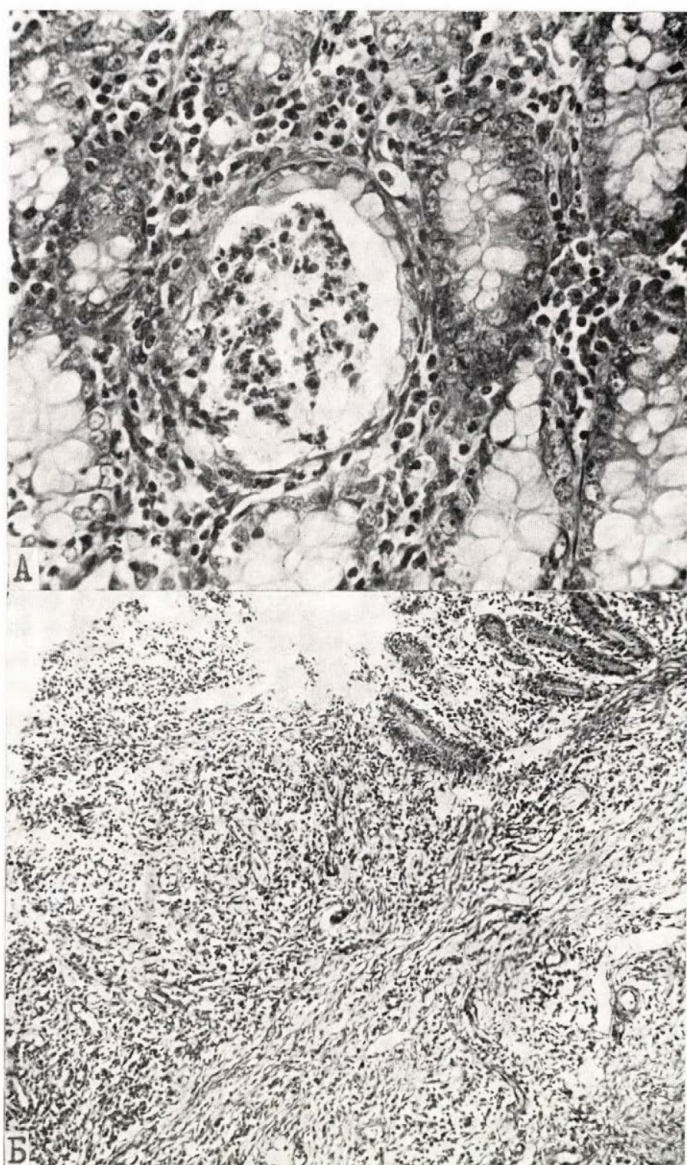


Рис. 5. Фрагменты толстых кишок обезьян через 5 дней после курса скармливания стрептомицин-зависимого штамма шигелл Зонне. Окраска гематоксилин-эозином. Увеличение 345х. А. Воспалительный экссудат в криптах. Б. Эрозия в слепой кишке.

Таблица I

Результаты проверки специфической безопасности вакцинных

Штаммы шигелл	Кератоконъюнктивальная проба					
			Гистологические исследования			
			Кератит	Конъюнктивит	Проникнове- ние в клетки	Размножение в клетках
Strp-d штамм шигелл Флекснера 2a 1605/3	—	—	—	±	(±)	—
Strp-d штамм шигелл Зонне Z	—	—	—	±	(±)	—
Спонтанный мутант шигелл Флекснера 2a 516 M	—	—	—	±	±	—
Спонтанный мутант шигелл Зонне 6S	—	—	—	±	±	—
Вирулентный штамм шигелл Флекснера 2a	#	#	#	#	#	#
Вирулентный штамм шигелл Зонне	#	#	#	#	#	#

— степень воспалительной реакции, проникновения в клетки и внутриклеточного размножения, наблюдающиеся после введения вирулентных штаммов шигелл

(±) — шигеллы не постоянно обнаруживаются в клетках эпителия

± — единичные шигеллы постоянно обнаруживаются в клетках эпителия

чительном количестве, но закономерно, обнаруживались в клетках покровного эпителия (рис. 7). Эти данные также свидетельствуют о более высокой остаточной вирулентности шигелл спонтанных мутантов.

В сводной таблице (ри. 8) приведены данные этих исследований, которые указывают на различия в поведении вакцинных штаммов в использованных моделях.

Полученные с помощью различных лабораторных тестов данные о специфической безопасности вакцинных штаммов позволили рекомендовать приготовленные из них вакцины для изучения их реактогенности и эпидемиологической эффективности на ограниченных контингентах людей. Эти данные позволили также принять минимальные требования к допус-

штаммов шигелл Флекснера и зонне в лабораторных тестах

Легочная модель на белых мышах		Скармливание обезьянам			Размножение в клетках культур in vitro		
Размножение шигелл в легких мышей	Гибель мышей	Клинические проявления заболевания	Гистологические исследования		HeZa	Hep-2	Fl
			Патологические изменения в кишечнике	Проникновение шигелл в клетки			
—	—	—	—	(±)	—	—	—
—	—	—	±	(±)	—	—	—
—	—	—	±	±	—	—	—
—	—	—	±	±	—	—	—
#	#	#	#	#	#	#	#
#	#	#	#	#	#	#	#

тимой остаточной вирулентности вакцинных штаммов и специфической безопасности полученных из них вакцин. В качестве теста для контроля специфической безопасности производственных серий живых дизентерийных вакцин принята кератоконъюнктивальная проба на морских свинках. Возможно, что в дальнейшем эта модель будет заменена или дополнена моделью шигеллезной пневмонии у мышей. Последняя не уступает по своей чувствительности кератоконъюнктивальной пробе, и к тому же она проводится на более дешевых животных.

Для оценки специфической безопасности и уровня «остаточной вирулентности» вакцинных штаммов принята кератоконъюнктивальная проба на морских свинках и скармливание обезьянам Макака резус. По нашему мнению, тщательная проверка вакцинных штаммов (перед использованием их для производства вакцин) и контроль полученных из них вакцин на специфическую безопасность обеспечат выпуск безвредных для людей дизентерийных вакцин.

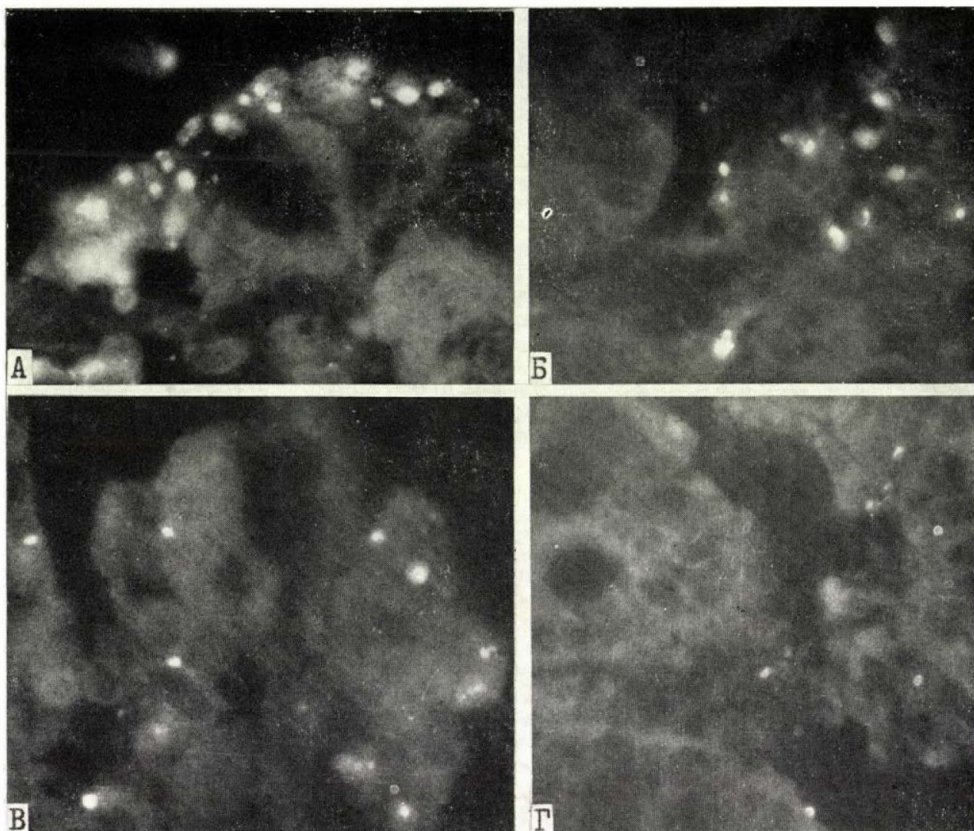


Рис. 6. Эпителий участков толстых кишок обезьян через 2 дня после окончания курса иммунизации стрептомицин-зависимым штаммом шигелл Флекснера 2а. Люминесцентно-серологический метод. Увеличение 900х. А. Скопления шигелл в эпителиальных клетках у обезьяны, зараженной вирулентным штаммом шигелл Флекснера 2а. Б. Единичные шигеллы в редких клетках покровного эпителия.

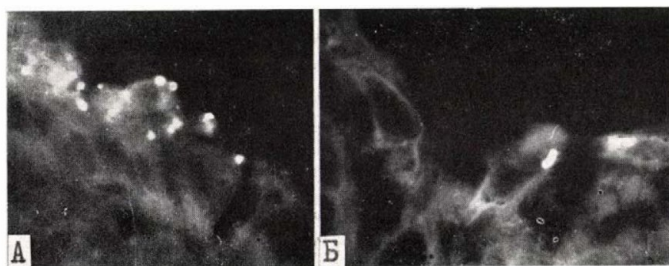


Рис. 7. То же после скармливания спонтанных мутантов шигелл Флекснера 2а. А. Множество микробов в поверхностных эпителиальных клетках после заражения вирулентным штаммом. Б, В и Г. Кишечник обезьян, иммунизированных спонтанным мутантом шигелл Флекснера 2а. Обнаруживается небольшое количество шигелл в ряде эпителиальных клеток. Б. Через 2 дня после окончания курса иммунизации. В. Через 5 дней. Г. Через 10 дней.

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IMMUNITY TO KERATOCONJUNCTIVITIS SHIGELLOSA IN RABBITS AFTER ORAL IMMUNIZATION WITH *SHIGELLA SONNEI* FREE ENDOTOXIN

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Summary. Orally administered free *Shigella sonnei* phase I endotoxin protected rabbits against keratoconjunctivitis shigellosa and produced IgG and IgM antibodies. There was no correlation between the protective activity and haemagglutinin titre.

In 1969, SLOPEK *et al.* [1] showed that free endotoxin (FE) was released from *Shigella sonnei* phase I, and that FE preparations obtained from a cell-free filtrate possess biological properties similar to those of endotoxin extracted from bacterial cell walls of *S. sonnei* phase I [2, 3]. Subsequently it was shown [4] that FE was strongly immunogenic. Its intravenous injection caused an increase in the level of humoral antibodies.

The aim of the present study was to establish whether FE administered orally to rabbits stimulated the production of specific antibodies [4] and whether it protected the animals against keratoconjunctivitis shigellosa (KC) induced by virulent forms of *S. sonnei* [5]. Results are shown in Figs 1, 2 and 3.

Each group of animals consisted of 5 rabbits. The results for each group are mean values obtained in 5 rabbits. In the experiments the same FE preparation was used in the same dose, but the administration schedule and the interval between the last immunization and the KC test varied from one group to the other.

In Fig. 1 the results obtained in two groups are presented. In group A, the animals received 2 mg of purified FE on a single occasion. The rabbits of group B were fed nine times, every 24 hr, with the same amount of FE. As shown in Fig. 1, the free endotoxin stimulated production of specific antibodies. Even after single doses of FE, the presence of haemagglutinins was observed. The KC test was performed 15 days after the last oral immunization. An increase in IgM and IgG was observed when virulent forms of *S. sonnei* were used to perform the KC test. However, the presence of specific humoral antibodies did not protect the animals against KC shigellosa. Although the intensity of KC was lower in the immunized animals than in the control group, and especially so in group B, further experiments showed that the acquired immunity was only short-lived.

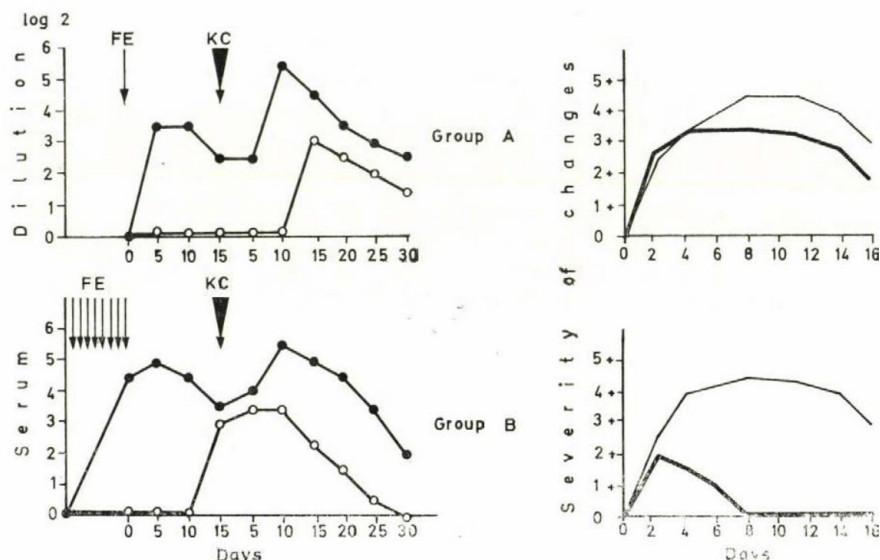


Fig. 1. Titre of haemagglutinins depending on the number of free endotoxin doses and the development of KC shigellosa. Intensity of KC in the control animals and in the rabbits immunized with FE. The KC test was performed 15 days after the last dose of FE. ●—● haemagglutinin titre in rabbit sera without 2-mercaptoethanol; ○—○ haemagglutinin titre in sera treated with 2-mercaptoethanol; ——— keratoconjunctivitis (KC) shigellosa after treatment of rabbits with free endotoxin (FE); ——— KC shigellosa in control rabbits

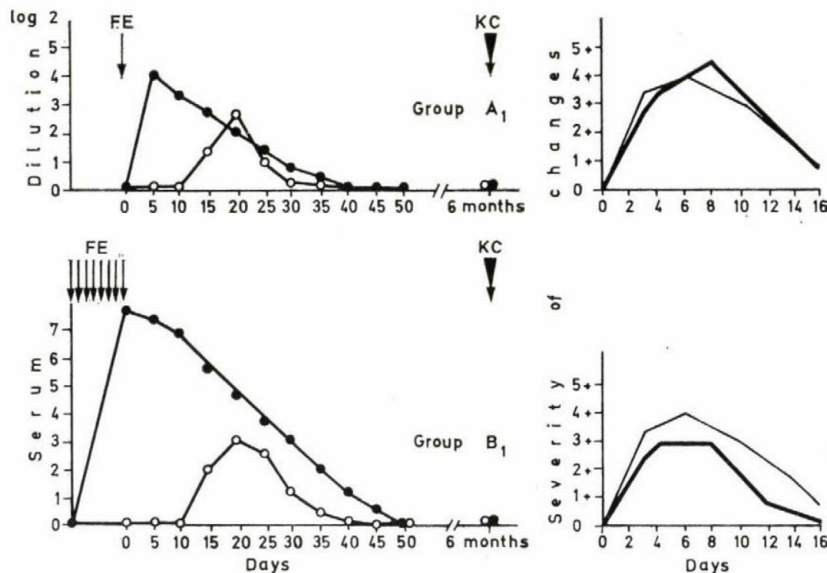


Fig. 2. Haemagglutinin titres depending on the number of FE doses. The KC test was performed 6 months after the last immunization. ●—● haemagglutinin titre in rabbit sera without 2-mercaptoethanol; ○—○ haemagglutinin titre in sera treated with 2-mercaptoethanol; ——— keratoconjunctivitis (KC) shigellosa after free endotoxin (FE) treatment; ——— KC shigellosa in control rabbits

The results of haemagglutinin titre determinations and the intensity of KC in rabbits immunized in the same way as in groups A and B are shown in Fig. 2. In this case, however, the KC test was performed 6 months after the last dose of FE. As shown in Fig. 2, the highest haemagglutinin titre was observed within 10 days after feeding the endotoxin. This was followed by a slow decrease, and after 6 months no haemagglutinating activity was found

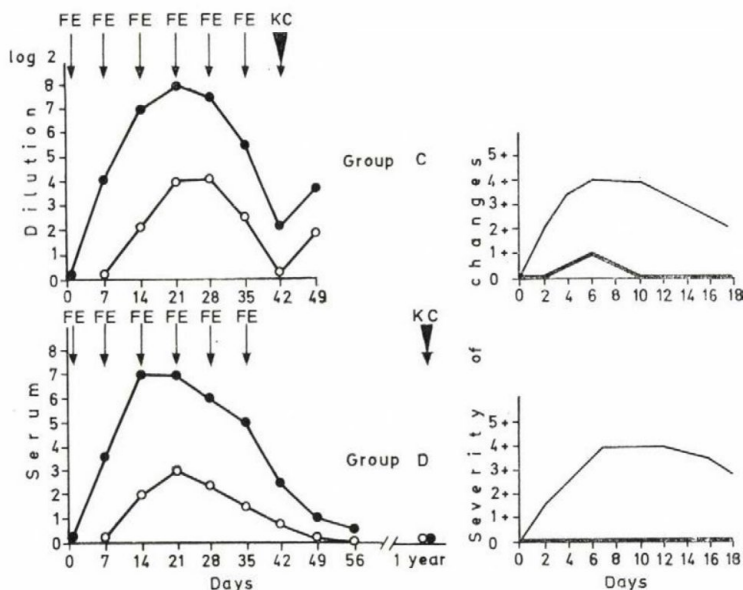


Fig. 3. Haemagglutinin titres depending on the interval between successive immunizations. The KC test was performed 6 months (group C) and 1 year (group D) after the last immunization. ●—● haemagglutinin titre in rabbit sera without 2-mercaptoethanol; ○—○ haemagglutinin titre in sera treated with 2-mercaptoethanol; — keratoconjunctivitis (KC) shigellosa after free endotoxin (FE) treatment; — KC shigellosa in control rabbits

in the immunized animals. The intensity of KC was the same in the immunized and in the control rabbits. These results showed that the immunity acquired after nine daily immunizations was short-lived.

Fig. 3 presents the results of immunization of rabbits in groups C and D. The animals were immunized orally 6 times, with one dose every week. Group C differed from group D in the interval between the last immunization and KC test. In this case an increased production of IgG and IgM occurred. The highest antibody level was observed between the second and fourth week of immunization. Prolongation of immunization caused a decrease in the antibody titre. Despite a low level of humoral antibodies, of 5 rabbits 4 were resistant in the KC test performed one week after the sixth immunization.

The KC test performed in rabbits of group D one year after the last immunization showed that all animals were resistant to *S. sonnei* while the control animals proved fully sensitive in the KC test. The protective activity of FE depended on the interval between successive immunizations.

Free *S. sonnei* phase I endotoxin introduced orally induced immunological response in rabbits and protected the animals against KC shigellosa. The free endotoxin caused the appearance of IgG and IgM class antibodies. Immunity to KC shigellosa was highest when weekly intervals were employed. No correlation was found between the serum haemagglutinin titre and the protective activity to KC.

Free endotoxin, owing to its immunogenic and immunizing properties, might prove useful for protective immunization of humans against bacterial dysentery.

Acknowledgement. We are indebted to Dr. E. ROMANOWSKA for supplying free endotoxin preparation of *S. sonnei* phase I.

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IMMUNOGENIC VALUE OF LIVE, INACTIVATED AND CELL-FREE DYSENTERY VACCINES

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Summary. The immunogenic value of different types of dysentery vaccine (*Shigella flexneri* and *Shigella sonnei*) was tested on the guinea-pig eye. Groups of 30 animals were immunized by the conjunctival route for 14 days and then, two weeks after the last application of the vaccine (or extract), infected with virulent strains. The best immunogenicity was shown by the live vaccines, especially by the non-dependent vaccine strains and the second best by the streptomycin-dependent strains. The inactivated vaccine ensured a reduced immunogenicity and the bacterial extracts (Boivin—Mesrobeanu-type antigen and thermolabile antigen) produced no local immunity. A good homologous and heterologous (anti-Sonne) immunogenicity was observed with live Flexner vaccine, whereas live Sonne vaccines only gave a good homologous immunity.

Investigations carried out in several countries into the immunogenic value of different types of dysentery vaccine prepared from inactivated germs or microbial extracts, have shown their poor preventive efficiency, both experimentally and in epidemiological tests [1–4]. During the last few years these investigations have been extended to some live vaccines [5, 6] which were then found to have an appreciable preventive and therapeutical effect in both laboratory animals [7, 8] and human communities [9–13].

There being no comparative studies of the *Shigella* vaccine strains used in live vaccines and of the inactivated vaccine and microbial extracts, it was considered of interest to report on the results of our investigations carried out with these products in the guinea pig.

The following vaccines and microbial extracts were used. 1. Live vaccines: 1.1. *Shigella flexneri* 2a T₃₂ vaccine (Cantacuzino Institute); 1.2. *S. flexneri* 2a Sm-d (D. MEL, Yugoslavia); 1.3. *S. sonnei* Lac⁺ (Cantacuzino Institute); 1.4. *S. sonnei* Sm-d (D. MEL, Yugoslavia). 2. Inactivated vaccines: 2.1. *S. flexneri* 2a T₃₂ vaccine inactivated at 60°C for 1 hr; 2.2. *S. sonnei* Lac⁺ vaccine inactivated at 60°C for 1 hr. 3. Bacterial extracts: 3.1. Boivin—Mesrobeanu type *S. sonnei* antigen; 3.2. thermolabile *S. sonnei* antigen. Vaccines 3.1 and 3.2 were prepared in the Institute of Experimental Epidemiology, Wernigerode, GDR.

Groups of 30 guinea pigs from the same breed, weighing 200 to 250 g, were immunized by the conjunctival route in both eyes by daily application for 14 days of the bacterial vaccines in doses of 2×10^9 germs, or the extracts

in doses of 0.05 mg equivalent to the amount of germs used. The vaccines and extracts were applied in a 0.005 ml suspension or solution with a Luer precision syringe.

Fourteen days after the last application of the vaccine or extract, the animals were infected by the conjunctival route with virulent germs in amounts ranging from 1 ID₆₅ to 1 ID₁₀₀ (infectious dose producing keratoconjunctivitis in 65 to 100% of the guinea pig eyes). The *S. flexneri* 2a 1678

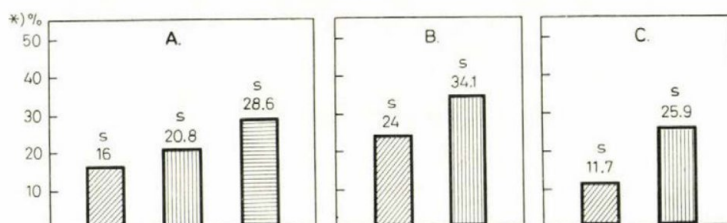


Fig. 1. Incidence of positive KC test in guinea pigs immunized locally with different *S. flexneri* vaccines (A, B, C = experiments at different dates). * = Percentage of diseased guinea-pig eyes referred to the number of guinea pig eyes 100% susceptible to the challenging dose applied. A, B = homologous challenge (*S. flexneri* 2a); C = heterologous challenge (*S. sonnei* S); S = statistically significant difference against controls ($P < 0.1$)

-  Live *S. flexneri* 2a T₃₂ vaccine capable of multiplication;
-  live *S. flexneri* 2a Sm-d vaccine;
-  inactivated *S. flexneri* 2a T₃₂ vaccine

and *S. sonnei* "S" 96 strains were used for challenge. The challenging doses were established previously by repeated comparative determinations, as well as in control groups of non-immunized guinea pigs infected simultaneously with the animals immunized previously by the conjunctival route.

The immunized guinea pigs which did not exhibit symptoms of keratoconjunctivitis after infection with virulent germs were reinfected heterologously after an 8-day interval. The results were analyzed statistically by the χ^2 test.

Fig. 1 gives the incidence of the positive keratoconjunctivitis (KC) tests in the guinea pigs immunized by the conjunctival route with different *S. flexneri* vaccines and subsequently challenged with a homologous (*S. flexneri* 2a) or heterologous (*S. sonnei*) strain. In each experiment in which infection was performed either with the homologous (Fig. 1A and B) or the heterologous strain (Fig. 1C), the lowest incidence of KC was noted among the guinea pigs vaccinated with the live *S. flexneri* 2a T₃₂ vaccine and the live streptomycin-dependent vaccine, and lastly with the inactivated vaccine.

The lowest incidence of KC after homologous infection (Fig. 2A and B) was likewise observed after the live *S. sonnei* Lac⁺ vaccine and the live streptomycin-dependent *S. sonnei* vaccine. The inactivated *S. sonnei* vaccine conferred a poor anti-infectious protection. The lipopolysaccharide antigen and the thermolabile antigen proved inefficient.

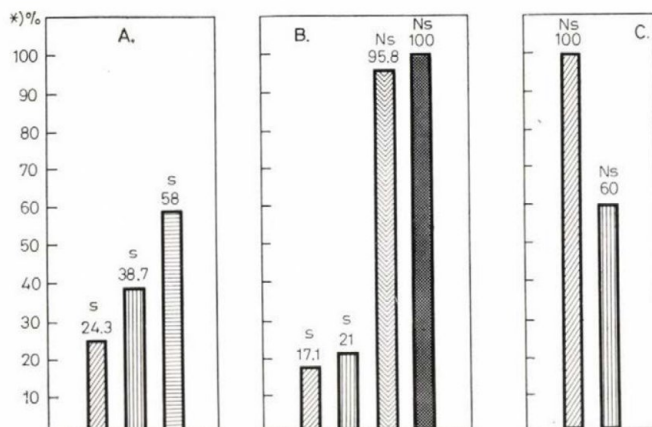


Fig. 2. Incidence of positive KC test in guinea pigs immunized locally with different *S. sonnei* vaccines (A, B, C = experiments at different dates). * = Percentage of diseased guinea pig eyes referred to the number of guinea pig eyes 100% susceptible to the challenging dose. A, B = homologous challenge (*S. sonnei* S); C = heterologous challenge (*S. flexneri* 2a); S = statistically significant difference against the controls ($P < 0.1$ or < 1); Ns = statistically non-significant difference against the controls. [diagonal lines] Live *S. sonnei* Lac⁺ vaccine capable of multiplication; [horizontal lines] live *S. sonnei* Sm-d vaccine; [vertical lines] inactivated *S. sonnei* Lac⁺ vaccine; [dots] *S. sonnei* thermolabile antigen; [solid black] Boivin-Mesrobian-type *S. sonnei* antigen

In the guinea pigs immunized with the *S. sonnei* Lac⁺ vaccine there was no protective activity against heterologous infection with *S. flexneri* (Fig. 2C), and a reduced, statistically insignificant protective activity after vaccination with the streptomycin-dependent strain.

The highest χ^2 values corresponding to the highest statistically significant differences as compared to the non-vaccinated control animals were found in the guinea pigs vaccinated with non-dependent *Shigella* strains then, in decreasing order, those vaccinated with streptomycin-dependent strains and the inactivated vaccine. Statistically significant differences were likewise observed between the live vaccines and the extracts, and between the non-dependent live *S. sonnei* Lac⁺ vaccine and the inactivated *S. sonnei* vaccine.

According to the above data, the best immunogenicity in the guinea pig is ensured by live vaccines, especially the non-dependent vaccinal strains *S. flexneri* 2a T₃₂ and *S. sonnei* Lac⁺ and the second best, the streptomycin-dependent strains.

The inactivated vaccine gives a reduced immunogenicity and the bacterial extracts (Boivin-Mesrobeanu-type antigen and thermolabile antigen) produce no local immunity, in agreement with previous data [14] that showed the same results for the Boivin-Mesrobeanu-type antigen extracted from *S. flexneri*.

Similarly, a good homologous and heterologous (anti-Sonne) immunogenicity was observed with the live *S. flexneri* vaccine, whereas the live *S. sonnei* vaccines only gave a good homologous immunity.

These findings are in agreement with the epidemiological data concerning the low preventive effect of certain *Shigella* extracts [1, 2], in contrast to the favourable effect obtained with the live vaccines [9-13]. The reduced immunity of the inactivated vaccines can only partly be extrapolated to man if we bear in mind that the ratio of the amount of antigen to the immunized surface, obtained in the course of the conjunctival vaccination test in the guinea pig, can only be realized by vaccine doses too large to be administered in practice. From this point of view, only the live vaccines capable of multiplication present a possibility of obtaining in the intestines an increased amount of antigen and, therefore, an improved antigen/immunized surface ratio.

In addition, apart from anti-infectious immunity ensured by the conjunctival immunization of the guinea pig and of other factors that interfere with the prophylactic effect in man, there is also an ecologic effect of the multiplying vaccinal strain, demonstrated *in vitro* by substitution of homologous with heterologous virulent strains [15].

The dissimilar anti-infectious response to different types of shigella antigens obtained by conjunctival vaccination of the guinea pig, might offer in the course of future investigations a possibility of selecting some highly immunogenic live *Shigella* vaccine strains.

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LIVE ORAL SHIGELLA VACCINE: VACCINATION SCHEDULE AND THE EFFECT OF BOOSTER DOSE

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Summary. (i) Pretreatment with sodium bicarbonate seems to be essential in attaining protection with streptomycin-dependent orally administered live *Shigella* vaccine. (ii) Protection against enteric infection can be achieved applying 5 or 4 doses, at 2 and 3 day intervals, respectively. The same result can be achieved by 3 doses at four day intervals, if streptomycin is administered simultaneously with the vaccine. (iii) The duration of protection conferred by the live oral vaccine lasted in children more than 6 but less than 12 months. (iv) Booster doses fed in three consecutive years after the primary vaccination enhanced the resistance to the disease in the same degree as did the primary vaccination.

For several years we studied the protective capacity of orally administered avirulent *Shigella* strains against bacillary dysentery in groups of men and children exposed to natural infection. As an avirulent strain, we are using streptomycin-dependent (Sm-d) mutants of *Shigella sonnei* and of various types of *Shigella flexneri*. As with all vaccines, the primary criterion of an attenuated strain is a high degree of safety and protective potency. Both these criteria have already been studied [1–4]. The present report deals with problems related to the use of our streptomycin-dependent strain as a live oral vaccine against bacillary dysentery.

In our first experiments we often failed to recover the vaccine strain from stools of the vaccinated volunteers. The mean duration of shedding, regardless of the dose of vaccine administered, amounted approximately to 0.8 days (range, 0 to 2 days). In contrast, the frequency of positive isolations increased when sodium bicarbonate was given prior to vaccination, and the mean duration of shedding amounted to three days (range, 0 to 6 days). Table I shows the effect of sodium bicarbonate on recovery of the vaccine strain from the faeces of the vaccinees. Sodium bicarbonate was instrumental in enhancing the gastro-intestinal transit of viable organisms. For this reason it was decided to use sodium bicarbonate prior to vaccination.

The mechanism involved in the effect of bicarbonate was investigated in adults. It was shown that the majority of volunteers had acid gastric contents, prior to bicarbonate below pH 2.0 and in such environments shigellae hardly survive. After administration of 2 g sodium bicarbonate the stomach contents was buffered to pH 6.0 or higher. The results of HORNICK's investi-

Table I

Effect of pretreatment on the isolation of streptomycin-dependent *S. flexneri* 2a vaccine strain from stools of vaccinated adults
Vaccine dose: 50×10^9 organisms

Pretreatment	No. of subjects	Subjects with isolation	
		No.	per cent
None	105	19	(19.0)
2 g NaHCO_3	112	101	(90.1)

gations with *Vibrio cholerae* support the hypothesis that bicarbonate acts by protecting organisms from the acid gastric contents [5].

At first, the vaccine was applied in a total of five doses, given every third day. The two day intervals were chosen in order to ensure an uninterrupted contact of the gastrointestinal tract of the vaccinees with the Sm-d shigellae. In other words the subsequent doses should be given before elimination of shigellae from the former dose. The five doses were chosen in order to maintain this contact for 14 days, a period sufficiently long to serve as an efficient immunogenic stimulus. Results obtained in field trials showed a type-specific protection ranging between 84% and 100%.

Somewhat lower or a similar degree of protection was achieved in adults and children, when the vaccine was given in four doses at intervals of three days. The total period during which the Sm-d shigellae persisted in the intestinal tract was the same as before. With three doses no protection could be achieved either at three or at four day intervals. However, in two consecutive field trials with adults three doses at 4 day intervals induced protection, if streptomycin was added to the reconstituted lyophilized vaccine prior to its administration.

Studies on several hundreds of adults and children repeated many times with different doses of streptomycin have shown that

— streptomycin can be administered simultaneously with the vaccine without untoward effects to the vaccinated adults and children;

— streptomycin given simultaneously with the vaccine prolongs the length of shedding of Sm-d shigellae from the intestinal tract from three to four days;

— when streptomycin is added to the vaccine, the number of Sm-d organisms is significantly increased in the stools of the vaccinees, as compared with those vaccinated without streptomycin. However, a multiplication of Sm-d organisms in the intestinal tract of the vaccinees could not be established.

These results were obtained by adding to each dose 50 mg or less streptomycin. Even 750 mg streptomycin per dose can safely be given to adults

without any adverse reaction. Studies are now being conducted with higher doses of streptomycin.

The mechanism of the streptomycin effect may be subject to various interpretations such as an enhanced multiplication in the presence of streptomycin; suppression of the normal intestinal flora; or a certain toxic action of streptomycin upon the host. Whatever the mechanism, the fact remains when streptomycin is added to the vaccine, three doses of it are inducing a significant protection against enteric infection with shigellae.

In our studies we often had difficulties with children to make them ingest the pretreatment solution containing 2.0 g sodium bicarbonate in water. We felt that it would be much easier to apply the vaccine without pretreatment, *i.e.*, to reduce the vaccination process to one single operation. The attempts at a more practicable way of vaccination resulted in adoption of a single mixture consisting of milk, sodium bicarbonate, streptomycin and Sm-d organisms.

The efficacy of this method was tested in adults. They ingested in a single dose 50×10^9 organisms with either 2.0 g sodium bicarbonate as pretreatment, or the organisms were given in milk with sodium bicarbonate and streptomycin, without pretreatment. The results outlined in Table II attest

Table II

Effect of pretreatment on the isolation of streptomycin-dependent S. flexneri 2a vaccine strain from stools of vaccinated adults

Pretreatment 5 min. prior to vaccination	Vaccination with 50×10^9 Sm-d organisms	No. of subjects	No. of subjects shedding the vaccine strain
2 g NaHCO_3	in saline	26	24
None	in milk with 1.5 g NaHCO_3 and 20–50 mg streptomycin	26	26
None	in saline	26	5

the usefulness of the milk mixture. As it can be seen, vaccine organisms were isolated from 24 stool cultures of 26 adults ingesting sodium bicarbonate, and from 26 of 26 adults given a single dose of vaccine–sodium bicarbonate–streptomycin in milk. Only 5 of 26 adults having no pretreatment shed the vaccine strain.

Encouraged by these results, a study of the efficacy of the vaccine applied in the above way was undertaken in adults in a hyperendemic area.

A total of 367 adults received *S. flexneri* 2a vaccine and 358 received *S. sonnei* vaccine. Feeding of one or the other type of vaccine was arranged randomly in different dormitories. In each dormitory, about 50% of randomly

selected adults were vaccinated in a blind fashion against *S. flexneri* 2a, while the other half received vaccine against *S. sonnei*. The vaccine was applied in milk, together with sodium bicarbonate, streptomycin and the Sm-d organisms, giving 50, 60 and 70 $\times 10^9$ live organisms in the first, second and third dose, respectively, with four day intervals between the doses.

No reaction whatsoever was noted in any of the 725 vaccinated adults.

During the follow-up, which lasted from June 1 to August 31, the population under study was struck by a severe waterborne outbreak. A total of 93 cases of bacillary dysentery was confirmed bacteriologically; of these 89 had been caused by *S. flexneri* serotype 2a. Table III shows that the morbidity

Table III
Morbidity observed in 1972 (1 June–31 August)

Diagnosis	No. of subjects vaccinated with	
	<i>S. flexneri</i> 2a	<i>S. sonnei</i>
<i>S. flexneri</i> 1	1	—
<i>S. flexneri</i> 2a	21 (5.7%)	68 (18.9%)
<i>S. flexneri</i> 3	—	1
<i>S. sonnei</i>	1	—
<i>S. boydi</i>	—	1
Diarrhoea only	11	12
All cases	34	82
Healthy	333	276
Total	367	358

due to *S. flexneri* 2a observed in the non-protected group against *S. flexneri* 2a (68 cases, representing an incidence of 18.9%) was significantly higher than that observed among adults vaccinated with type 2a vaccine (21 cases, representing an incidence of 5.7%). The difference in frequency was highly significant ($P < 0.001$), and the degree of protection amounted to 70%.

The degree of protection induced by the vaccine fed in milk was lower than that obtained in all our field trials in the last 10 years. Two explanations may be offered for the low protection. One, that the vaccine given in the milk mixture cannot afford a higher protection. Second, that the low protection achieved could be ascribed to the heavy infective dose which certainly occurred in this outbreak. The severity of the epidemic can be seen in Fig. 1, which shows the distribution of *S. flexneri* 2a cases in five-day periods. A similar graph with daily distribution would show three clearly distinguishable waves, caused probably by a heavy periodical contamination of the water. This may agree with the results of DUPONT *et al.* using Sm-d shigellae [6].

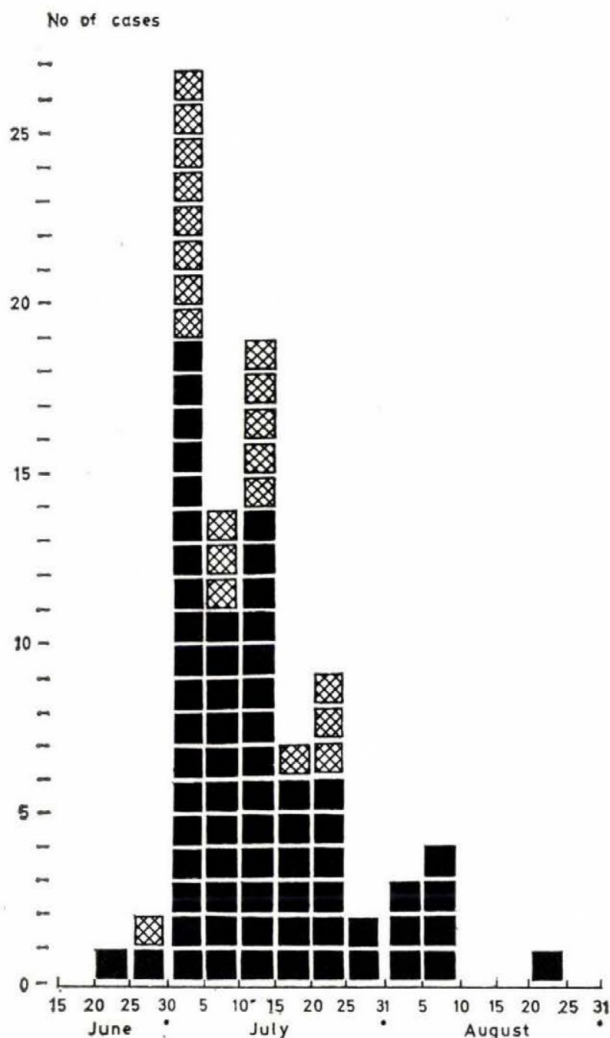


Fig. 1. Distribution of *S. flexneri* 2a cases at five day intervals (1972, June 1—August 31). Hatched columns: protected; solid columns: control

A field trial which is just in course might clarify the reason for the low protection obtained.

From a total of 1643 children primarily vaccinated with four doses during the 1969 field trial, nearly 800 were revaccinated in 1970, in order to obtain information as to the protection offered by a booster dose given one year after the primary vaccination. The rest of the children were also controlled to get information as to the duration of protection by the vaccine fed in 1969.

From the results it has been concluded that the protection conferred by the primary vaccination lasted longer than 6 months but faded within 12

months, and that a single booster dose given one year after the primary vaccination reinforced the protection similarly as a complete primary vaccination.

The same children were again revaccinated with a booster dose in 1971 and again in 1972. Results of the follow-up during 1970, 1971 and 1972 are shown in Table IV, which presents the cumulated morbidity of children vacci-

Table IV
Cumulated morbidity in children during the observation periods in 1970, 1971 and 1972

Diagnosis	No. of children vaccinated in 1969			
	and revaccinated in 1970, 1971 and 1972 against		against	
	<i>S. flexneri</i> 2a	<i>S. sonnei</i>	<i>S. flexneri</i> 2a	<i>S. sonnei</i>
<i>S. flexneri</i> 2a	1	12	15	14
<i>S. sonnei</i>	15	1	17	18
Other serotypes	21	21	29	28
Diarrhoea only	119	116	145	151
All cases	156	150	206	211
Healthy	214	212	204	205
Total	370	362	410	416

nated in 1969 and boosted in 1970, 1971 and 1972, and of children without boosters. Morbidity due to *S. flexneri* 2a and *S. sonnei* in the protected group was 0.27% (2/732) as against 3.68% (27/732) in the unprotected group. The difference was highly significant and the degree of protection conferred by the booster doses fed in the three consecutive years was 90.4%. In contrast, in the children without booster, the morbidity rates were comparable to those in the unprotected groups.

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USE OF A NEW PHAGE PREPARATION IN PROPHYLAXIS AND TREATMENT OF SHIGELLOSIS

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Summary. The effectiveness of phage preparation in the prophylaxis and treatment of shigellosis has been examined. Mass use of phage preparation is economically advantageous as it replaces the expensive and often ineffective treatment of dysentery with sulphonamides and antibiotics. An additional advantage of phage therapy over sulphonamides and antibiotics is that it acts specifically on dysentery organisms without destroying the normal flora of the human digestive tract.

The wide use of sulphonamides and antibiotics as the drugs of choice in the treatment of bacterial dysentery was quickly followed by the emergence of drug resistant strains. It has been shown that the resistance of *Shigella* strains is determined by the R factor, which is transmitted from cell to cell by conjugation [1].

In recent years, the incidence of *Shigella flexneri* and *Shigella sonnei* dysentery has increased in Poland [2, 3]. Prevention and treatment of the disease by sulphonamides and antibiotics has met with serious difficulties owing to the mass occurrence of resistant *Shigella* strains carrying the R factor [4–8]. We have therefore developed polyvalent phage preparations for *S. flexneri* and *S. sonnei*.

Selection of phages for polyvalent preparations was done on the basis of a thorough analysis of the results of phage typing of 460 *S. flexneri*, and 2127 phase I and phase II *S. sonnei* strains isolated in Poland in the years 1966 to 1971 [9, 10]. The *S. flexneri* phage preparation is a mixture of equal volumes of four bacteriophages: F2, F4, F6 and F10 with a lytic range (10^8 – 10^9 particles per ml) embracing all the *S. flexneri* phage types encountered in the material (Table I). The *S. sonnei* phage preparation is a mixture of equal volumes of four bacteriophages: SK2, SK5, SK7 and SG35 (10^8 – 10^9 particles per ml), which lysed all phage types distinguished among phase I and phase II of *S. sonnei* isolated in Poland (Table II).

Table I shows that most *S. flexneri* strains were sensitive to two or more of the phages. Strains sensitive to a single phage belonged predominantly to phage types 51 and 53 of the serotype 6 of *S. flexneri*. Among the shigella phages in the collection of our laboratory, no other phage was found to act on that serotype.

The situation was similar with the phase I and phase II *S. sonnei* strains. As seen in Tables II and III, most *S. sonnei* strains were lysed by three or four phages.

Table I

Lytic spectrum of phages of polyvalent phage preparation on S. flexneri phage types isolated in Poland

Phage type*	Bacteriophages				No. of strains
	F2	F4	F6	F10	
1	Cl	Cl	Cl	Cl	27
9	Cl	—	Cl	—	3
10	Cl	—	Cl	—	3
12	—	Cl	Cl	^s Cl	46
15	—	Cl	Cl	—	48
19	—	Cl	Cl	—	12
20	—	++	^s Cl	Cl	3
26	—	—	Cl	Cl	13
32	^s Cl	Cl	—	Cl	17
35	^s Cl	Cl	—	Cl	14
38	Cl	—	—	—	7
41	Cl	—	—	—	5
42	Cl	—	—	—	5
43	—	Cl	±	Cl	110
48	—	—	±	Cl	14
49	—	—	±	Cl	2
51	—	—	—	Cl	2
53	—	—	—	Cl	129

* According to SLOPEK *et al.* [9]

Explanation for Tables I—III

Cl = confluent lysis

^sCl = semiconfluent lysis

+++ = 80 or more plaques

++ = 40–80 isolated plaques

± = 20–40 isolated plaques

± = single plaques

— = negative reaction

The finding that most *S. flexneri* and *S. sonnei* strains were sensitive to at least two or more phages contained in the preparations practically excluded the appearance of phage-resistant mutants.

The effectiveness of the *S. sonnei* phage preparation was assessed in a number of children's institutions, where in the years 1970–1972 cases of dysentery had occurred. In these institutions, three faecal examinations revealed 120 carriers (18 adults among the personnel and 102 children from 3 months

Table II

Lytic spectrum of phages of polyvalent phage preparation on S. sonnei phase I phage types isolated in Poland

Phage type*	Bacteriophages				No. of strains
	SK2	SK5	SK7	SG35	
1	Cl	Cl	Cl	Cl	17
2	Cl	Cl	—	Cl	86
3	Cl	—	—	Cl	2
4	Cl	Cl	—	—	2
5	Cl	—	Cl	—	2
6	—	Cl	—	Cl	9
7	—	—	Cl	—	1
8	—	—	—	Cl	1

to 5 years of age) excreting *S. sonnei*, most of which were resistant to the sulphonamides and antibiotics commonly used in the treatment of dysentery.

Treatment of the carriers was done as follows. Before phage administration, adults were given 10 ml, children up to 7 years, 5 ml, and infants 2 ml of 5% sodium bicarbonate solution. The phage preparation was administered orally in a dose of 10 ml for adults and children, and 5 ml for infants, 1 hr before a meal twice daily for 7 days.

The first follow-up faecal examination was made 7 days after phage administration, and the second and third examinations were done at 1–3 day intervals thereafter.

As can be seen in Table IV, the first course of phage treatment resulted in a cure of the great majority of carriers, as evidenced by three negative faecal cultures. In 3 persons who failed to respond to the first treatment, the second course caused the shigellae to disappear from the stools. Unfortunately, in 4 persons the second course of treatment could not be performed because of an intercurrent influenza epidemic.

All the remaining children and personnel in the children's homes who had three negative faecal cultures were fed the phage preparation prophylactically once daily on 2–4 occasions at 5–7 day intervals. This prophylactic treatment was given to 1223 persons including 1003 children aged 3 months to 5 years and 220 adults. Among these persons no case of dysentery occurred during the next 6 months.

The results indicated that the polyvalent phage preparation was effective in the treatment and prophylaxis of bacterial dysentery, and in 1972 the Cracow Serum and Vaccine Laboratory produced more than 1000 litres for use in children's institutions.

Table III

Lytic spectrum of phages of polyvalent phage preparation on S. sonnei phase II phage types isolated in Poland

Phage type*	Bacteriophages				No. of strains
	SK2	SK5	SK7	SG35	
1	Cl	Cl	Cl	Cl	149
3	Cl	Cl	Cl	^s Cl	5
5	Cl	Cl	Cl	Cl	131
9	+++	Cl	Cl	Cl	6
13	Cl	Cl	Cl	Cl	12
18	Cl	Cl	—	Cl	4
19	Cl	Cl	—	Cl	114
24	Cl	Cl	—	Cl	100
26	Cl	Cl	Cl	Cl	99
31	Cl	Cl	Cl	Cl	65
33	Cl	Cl	Cl	Cl	9
34	—	Cl	Cl	^s Cl	3
35	—	Cl	Cl	—	4
43	Cl	Cl	Cl	Cl	117
44	Cl	Cl	Cl	Cl	15
47	Cl	Cl	—	Cl	21
48	Cl	Cl	—	Cl	81
50	+	^s Cl	—	Cl	4
52	Cl	Cl	—	Cl	7
53	Cl	Cl	—	Cl	7
54	Cl	Cl	—	Cl	6
56	Cl	Cl	—	Cl	8
57	Cl	Cl	—	Cl	19
63	Cl	Cl	+++	Cl	27
72	++	^s Cl	—	Cl	22
74	Cl	Cl	±	Cl	433
75	Cl	Cl	—	Cl	81
77	—	—	—	Cl	4
82	+	—	Cl	—	6
84	Cl	—	Cl	—	11
91	Cl	Cl	—	Cl	425
92	^s Cl	Cl	—	Cl	14
96	Cl	Cl	—	Cl	3

* According to SLOPEK *et al.* [10]; first set

Table IV

Successful treatment of carriers of S. sonnei with specific bacteriophage

No. of persons treated	No. of persons cured by	
	1st course of treatment	2nd course of treatment
120	113	3*

* In 4 persons the second course of treatment could not be performed because of an inter-current influenza epidemic

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БЕЗВРЕДНОСТЬ, РЕАКТОГЕННОСТЬ И ЭФФЕКТИВНОСТЬ ЖИВЫХ ДИЗЕНТЕРИЙНЫХ ВАКЦИН ФЛЕКСНЕРА В ЭКСПЕРИМЕНТАХ НА ЖИВОТНЫХ И В ОГРАНИЧЕННОМ ЭПИДОПЫТЕ НА ДЕТЯХ

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SERGEEV, V. V., ELKINA, S. I., SOLODNIKOV, YU. P., SHUSTER, B. YU., SERGEEVA, G. S., BULK, V. F., BOBKIN, S. V.: **Safety, reactivity and effectiveness testing of live *Shigella flexneri* vaccines in laboratory animals and children.** Streptomycin dependent mutants identical in cultural, biochemical and serological properties to the parent strains were isolated from *Shigella flexneri* 2a, 4a, 4b and *Shigella sonnei*. Vaccines produced from the mutants produced a definite immunity in guinea pig (eye test), in mice (shigella-pneumonia model) and in monkeys. Live *S. flexneri* 2a vaccine exhibited a low reactivity to laboratory animals and produced a type-specific immunity in children.

Резюме. Выделены и изучены стрептомицинзависимые мутанты Str. Flexneri 2a, 4a, 4b, *S. sonnei*, а также проведен полевой опыт на детях по выяснению эффективности живой лиофилизированной вакцины из стрептомицинзависимого мутанта *S. flexneri* 2a. По культуральным, биохимическим и серологическим свойствам свежевыделенные стрептомицинзависимые мутанты не отличаются от исходных штаммов. В экспериментах на морских свинках и обезьянах установлены морфологические критерии безопасности живых дизентерийных вакцин. Выраженная иммуногенность вакцин из стрептомицинзависимых мутантов показана на морских свинках (кератоконъюнктивальная проба), белых мышах (модель шигеллезной пневмонии) и обезьянах. Показана безвредность и низкая реактогенность живой дизентерийной вакцины *S. flexneri* 2a. Пероральное введение вакцины Флекснера 2a в полевом опыте на детях с последующей ревакцинацией вызвало формирование типоспецифического иммунитета.

В настоящее время можно считать положительно решенным вопрос о наличии иммунитета после перенесения дизентерийного заболевания.

Использование корпускулярных и химических вакцин против дизентерии, при парентеральном применении, как в экспериментах на чувствительных животных (морские свинки, обезьяны), так и при постановке эпидопытов на людях оказалось не эффективным. В последнее время успехи в изучении иммунитета при кишечных инфекциях, установление его клеточно-тканевого характера вновь вызвали интерес к иммунизации против этих инфекций пероральным путем.

В СССР, СФРЮ, СРР и США изучаются три типа вакцинных штаммов против дизентерии: 1) спонтанные мутанты; 2) стрептомицинзависимые мутанты; 3) аттенуированные гибриды (*Sh. flexneri*—*E. coli*). В ограниченных эпидопытах изучены лишь живые энтеральные вакцины из стрептомицин-

зависимых штаммов шигелл и показана выраженная их эффективность (Д. Мел, 1965—1972).

В настоящей работе кратко изложены суммированные данные по экспериментальному изучению стрептомицинзависимых мутантов шигелл и живых вакцин, приготовленных на их основе, а также результаты изучения эффективности вакцины Флекснера 2а в ограниченном эпизоите.

Нами были выделены стрептомицинзависимые мутанты шигелл Флекснера 2а, 4а, 4в, а также изучен мутант шигелл Флекснера 2а, переданный нам проф. Д. Мелом (СФРЮ). Кроме того, мы изучали стрептомицинзависимый мутант шигелл Зонне, полученный от проф. Линде из ГДР.

По культуральным, биохимическим и серологическим свойствам свежeweделенные стрептомицинзависимые мутанты шигелл не отличались от исходных вирулентных штаммов. По данным хроматографии на бумаге, аналитического ультрацентрифугирования и инфракрасной спектроскопии О-антиген свежeweделенных (или лиофилизированных после выделения) мутантов шигелл не имел существенных изменений по сравнению с О-антигеном исходных штаммов.

Практическая авирулентность мутантов шигелл Флекснера и Зонне была показана в экспериментах на белых мышах (модель шигеллезной пневмонии), морских свинках (модель Формала — голодающие морские свинки и Шереня — кератоконъюнктивальная проба), на обезьянах Макака Резус. Одновременное введение стрептомицинзависимых мутантов и стрептомицина (опыты с кератоконъюнктивальной пробой) не приводило к восстановлению вирулентных свойств.

Мутанты шигелл ревертировали *in vitro* к независимому от антибиотика состоянию с частотой 10^{-7} — 10^{-8} . Была изучена вирулентность 188 ревертантов стрептомицинзависимых мутантов шигелл Флекснера 2а и 144 ревертанта из штамма шигелл Зонне. Все ревертанты на модели кератоконъюнктивальной пробы и шигеллезной пневмонии оказались авирулентными. Произведено 945 высевок от животных (включая обезьян) и 1457 посевов кала людей на среды без антибиотика. Ни в одном случае нам не удалось обнаружить реверсии *in vivo*.

В экспериментах на морских свинках (кератоконъюнктивальная модель) и обезьянах установлены морфологические критерии безопасности живых дизентерийных вакцин. При гистологическом определении степени «остаточной вирулентности» мы считаем возможным допустить катаральный конъюнктивит, появляющийся через 6 часов после введения мутанта. Первичная апробация вакцинного штамма предусматривает обязательное изучение его на обезьянах. Критерии безопасности: отсутствие выраженной диареи и язвенных поражений, в том числе значительного катарального воспаления слизистой кишечника. Допускается неравномерное полнокровие, небольшой отек, немного точечных кровоизлияний.

Показано, что стрептомицинзависимые мутанты шигелл, в отличие от вирулентных штаммов, не обладают способностью размножаться в клетках тканевых культур Нер 2 и HeLa, что свидетельствует о возможности использования клеточных культур для изучения специфической безопасности вакцинных штаммов.

В экспериментах на животных установлена выраженная иммунологическая эффективность живых дизентерийных вакцин. Используя кератоконъюнктивную пробу на морских свинках, мы выяснили зависимость эффективности иммунизации от кратности введения вакцинных штаммов, установили длительность и специфичность иммунитета (табл. I—IV). Пяти-

Таблица I

Зависимость эффективности иммунизации морских свинок стрептомицинзависимыми мутантами шигелл Флекснера 2a от кратности введения культуры (средние данные 3 опытов)

Мутант	Заражающий штамм	Заражающая доза в LD ₅₀	Кратность иммунизации	Результат: не заболело глаз из общего числа иммуниз.	Количество иммунных глаз в %
1605—3 Str-d	1605—Str—S	3	1 5 8	0/12 34/40 33/35	0 85 91
1466—68 Str-d	1466—Str—S	3	1 5 8	0/7 12/14 13/16	0 85,7 81/2
1461—71 Str-d	1466—Str—S	3	5	3/17	17,5

Таблица II

Продолжительность иммунитета при иммунизации морских свинок мутантом S. flexneri 2a 1605—3—Str—d (пятикратная вакцинация с интервалом в 3 дня, заражающая доза — 3 ЛД₅₀)

Реакция глаза морской свинки	Результат заражения иммунизированных морских св.			
	Срок заражения после последней иммунизации в недел.			
	2	4	8	12
Отсутствие видимых изменений	17	4	1	—
Легкая форма кератоконъюнктивита	2	12	4	2
Тяжелая форма кератоконъюнктивита	1	4	15	18
Контроль (заболело глаз интактных м. св. из общего числа зараженных)	10/10	10/10	10/10	10/10

Таблица III

Эффективность реиммунизации морских свинок мутантом *S. flexneri* 2a 1605—3—Str—d.

Число реиммунизаций	Интервал между реиммунизациями	Реакция глаз морских свинок	Результат
4	30 дней	Отсутствие видимых изменений Легкая форма Тяжелая форма	18 2 —
2	60 дней	Отсутствие видимых изменений Легкая форма Тяжелая форма	13 5 2

Контроль (заболело кератоконъюнктивитом глаз интактных морских свинок из общего числа зараженных)

Таблица IV

Специфичность поствакцинального иммунитета

Мутант для иммунизации	Заболело из общего числа зараженных штаммом			
	<i>S. flexneri</i> 1605—Str—S	<i>S. flexneri</i> 1466—Str—S	<i>S. flexneri</i> 3 Str. S	<i>S. sonnei</i> Str—S
1606—3—Str—d	3/20 85%	5/20 75%	16/20 20%	19/20 5%
Контроль (интактные м. свинки)	8/8 0%	6/6 0%	7/8 12,5%	7/8 12,5%

кратное введение вакцинных штаммов шигелл Флекснера 2a и Зонне в конъюнктивальный мешок морских свинок являлось оптимальным — 80—85,7% глаз оказывались резистентными к введению 3LD₅₀ вирулентной культуры. Через 4 недели 65% животных были иммунными, через 6 недель — 25%. Заражение свинок спустя 12 недель после вакцинации показало отсутствие иммунитета. Мы предприняли попытку продлить резистентность глаз морских свинок за счет реиммунизации. Животные (40 морских свинок) были иммунизированы пятикратно стрептомицинзависимым мутантом *Sh. flexneri* 2a. Одну группу свинок ревакцинировали через 30 дней, вторую — дважды через 30 и 60 дней. Полученные данные свидетельствовали, что в результате ревакцинации морских свинок, проведенной через 30 дней, все животные оказались иммунными. Вторая реиммунизация также обеспечивала выраженную невосприимчивость.

Иммуногенность дизентерийных мутантов *Sh. flexneri* 2a 1605—3—Str—d, 1466—68—Str—d, 1466—71—Str—d и *Sh. flexneri* 4b 2851—2—Str—d была изучена на модели шигеллезной пневмонии у белых мышей.

Многokrатно повторенными опытами была определена оптимальная схема иммунизации: трехкратно в дозах 100 млн., 200 млн. и 300 млн. бактериальных тел, интервал между иммунизациями 3—4 дня. На 10 день после последней иммунизации мышей заражали интраназально вирулентной культурой в дозе 4—5 LD₅₀. На этой модели была изучена также эффективность убитых нагреванием вакцин (табл. V). Таким образом, при данной схеме

Таблица V
Эффективность трехкратной интраназальной иммунизации стрептомицинзависимыми мутантами и убитыми вакцинами шигелл Флекснера

Вакцины	Заражающий штамм	Результат (отношение числа погибших мышей к числу зараженных)	Средний % выживших мышей
1605—3— —Str—d	1605—Str—S	0/50 0/50 2/50	98,6
1466—71— —Str—d	1466—Str—S	23/50 28/50 35/50	42,6
2851—2— —Str—d	2851—Str—S	4/50 2/50 2/50	94,6
1605—Str—S гретая	1605—Str—S	18/50 21/50 24/50	58
1466—Str—S гретая	1466—Str—S	22/50 20/50 29/50	52

иммунизации был отмечен выраженный эффект от интраназального введения белым мышам стрептомицинзависимых мутантов (процент защиты равнялся 98,6—94,6). Не многим более 50% животных оказались защищенными в результате введения гретых вакцин. Низкая степень защиты была отмечена при введении мутанта 1466—71—Str—d, который длительно пересевался на средах со стрептомицином. Нами показано, что иммунизация мутантом Sh. Flexneri 1605—3—Str—d обусловила иммунитет видовой и типовой специфичности. Межтиповой и межродовой иммунитет выражен значительно слабее. Выраженность иммунитета была изучена также по степени уменьшения числа микробов вирулентного штамма в легких иммунизированных мышей по сравнению с контрольными. В опытах интраназальной иммунизации были определены эффективные иммунизирующие дозы вакцин, защищавшие от гибели 50% животных.

Для подтверждения иммунологической эффективности вакцины из стрептомицинзависимых мутантов шигелл Флекснера 2а и 4в были проведены опыты на обезьянах, у которых, как известно, эта инфекция в основном соответствует дизентерийному заболеванию у человека. Проведенные клинические, лабораторные и гистологические исследования свидетельствовали, что заражение обезьян вирулентной культурой шигелл Флекснера 2а и 4в вызывало типичное развитие дизентерийной инфекции. Пятикратное введение, энтерально, стрептомицинзависимого мутанта предохраняло обезьян от развития инфекционного процесса при последующем их заражении вирулентной культурой гомологичного штамма.

Нами была изучена динамика антителобразующих клеток, которые, как известно, являются показателем развития иммунного ответа. В работе был использован непрямой метод Ерни. Результаты количественного изучения иммунокомпетентных клеток у обезьян, иммунизированных пятикратно вакциной Флекснера 4в, показали, что наибольшее их число и максимум нарастания определялись в толстом кишечнике (слепой кишке). Именно в гомогенате слепой кишки по РПГА удалось обнаружить наиболее высокие титры антител (1 : 150 — 1 : 640), несколько меньшие титры отмечались в гомогенатах брыжеечных лимфоузлов, ткани селезенки и тонкой кишки. Введение обезьянам вакцины Флекснера 2а дало аналогичные результаты.

Безвредность вакцины Флекснера 2а была установлена путем дачи вакцины 21 волонтеру. Изучение реактогенности вакцины было проведено вначале на взрослых (опытная и контрольная группы по 140 человек). Были использованы бактериологические, иммунологические и клинические методы обследования. Опытная группа была разделена на 4 подгруппы, в каждой из которых устанавливались реакции на введение различных доз вакцины. Реакции отмечались в первые сутки (в основном в первые 6 часов) после приема вакцины. Они выражались в различных субъективных жалобах, среди которых большую часть составляли тошнота, рвота, урчание, нерезкая боль в животе. Следует добавить, что аналогичные жалобы имели место и в контрольной группе. Увеличение первой дозы вакцинации от 20 млрд. до 60 млрд. увеличивало число жалоб. В опытной группе число реакций составляло 12%, в контрольной — 11%.

В 1971 году в городе Ашхабаде была изучена реактогенность вакцины Флекснера 2а среди детей. Установление реактогенности вакцины проводилось поэтапно, начиная с детей более старшего возраста (14—10 лет, 10—7 лет, 7—5 лет и так далее до года). Учитывались реакции: рвота, температура до 37,5°, выше 37,5°, жидкий стул, боль в животе, головная боль. В опытной и контрольной группах было по 320 детей. Для первой вакцинации была подобрана группа детей 10—14 лет, которым вводилось 12,5 млрд. бактерий, реакции отсутствовали. Затем 42 детям этого же возраста ввели по 25 млрд.: — рвота была отмечена у 3 детей, у двух повышение температуры до 37,5°.

Несмотря на повышение дозировок в трех последующих вакцинациях до 50 млрд. реакции на введение не наблюдалось.

В группе детей 3—6 лет были изучены реакции на введение постепенно увеличивающихся доз первой вакцинации от 6,5 млрд. до 12,5 млрд. При этом у трех детей температура поднималась до $37,5^{\circ}$, у одного — рвота и у одного ребенка, страдающего диатезом, была обнаружена сыпь на теле. Температурная реакция и рвота исчезали через 24 часа после приема вакцины. В группе, принявших плацебо: у одного — рвота, у трех — температура до $37,5^{\circ}$ и у одного — боль в животе. Отсутствие реакций позволило увеличить последующие дозировки вакцины от 25 до 37,5 млрд. (последующие введения). В группе вакцинированных у одного ребенка наблюдалась температура до $37,5^{\circ}$, у одного — сыпь. При введении плацебо — у одного рвота, у другого — боль в животе. Таким образом, была показана крайне низкая реактогенность вакцины. При проведении эпидемиологического опыта были избраны следующие схемы вакцинации:

Дети 1—2 лет 6,5; 12,5; 25; 25; млрд. живых бактерий

Дети 2—3 лет 6,5; 25; 25; 25; млрд. живых бактерий

Дети 3—7 лет 12,5; 25; 25; 37,5; млрд. живых бактерий

Изучение эффективности вакцины осуществляли на основе строго контролируемого эпидопыта, при обеспечении равноценности опытной и контрольной групп на основе метода случайного выбора. Эпидемиологическая эффективность вакцины изучалась на детях организованных коллективов от 1 года до семи лет. В опытной и контрольной группах было по 6000 детей. Через 8 месяцев дети были ревакцинированы.

Результаты эпидопыта:

В течение 8 месяцев после окончания вакцинации в опытной группе заболело бактериологически подтвержденной дизентерией Флекснера 2а 8 детей, в контрольной — 23 ребенка. Гораздо более высокая эффективность была отмечена после ревакцинации: за 7 месяцев наблюдения (с апреля по октябрь 1972 года) заболеваемость дизентерией Флекснера 2а в опытной группе была в 9,4 раза ниже, чем в контрольной (при статистической достоверности полученных данных $t > 3$). В опытной группе заболело 5 человек, в контрольной — 47. При выраженной разнице в заболеваемости вакцинированных детей дизентерией гомологичного типа, частота заболеваний, вызванных шигеллами других видов и серотипов, в обеих группах была одинаковой, что свидетельствует о типоспецифическом характере иммунитета. Были изучены иммунологические показатели в РПГА у привитых детей (группа в 200 человек). На 20 день после окончания вакцинации подъем титра антител отмечался у 60% детей до 1 : 200 — 1 : 3200 (до вакцинации титр не превышал 1 : 100). Специфические гемагглютинины и 7S — иммуноглобулины сохранялись в сыворотках привитых детей до 3—3,5 месяцев. В дальнейшем отмечалось снижение уровня антител. Однако и через 5—6 месяцев после

вакцинации процент положительных РПГА превышал таковой до вакцинации. При ревакцинации в сыворотках иммунизированных наблюдались аналогичные изменения, однако антитела определялись в более высоких титрах.

Изложенные данные, по нашему мнению, позволяют сделать вывод, что оптимальной схемой иммунизации живой дизентерийной вакциной из стрептомицинзависимого мутанта шигелл Флекснера серотипа 2а является иммунизация в виде курса, состоящего из четырехкратной вакцинации, а затем ревакцинации через 3—3,5 месяца.

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EFFECTIVENESS OF ORAL INACTIVATED TYPHOID VACCINE

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Summary. Technical execution and the results of an extensive field trial with oral immunization against typhoid fever in Chile are reported. In two areas in Chile with a high incidence of typhoid fever, 50 000 persons aged 6 to 35 and 10 to 20 years were immunized with an oral inactivated typhoid vaccine developed and produced in the GDR. They received 300×10^9 killed typhoid and paratyphoid B bacteria for 3 days, i.e. a total of 540×10^9 typhoid and 360×10^9 paratyphoid B bacteria. The mentioned age groups were chosen because they had the highest morbidity among those tested. In the same areas, control groups were formed with a corresponding number of persons living under the same socio-economic and environmental conditions. Statistical analysis carried out 12 and 18 months after the first vaccination, and 12 months after revaccination, showed a significant protection amounting to 75-80% in both areas. The vaccine was tolerated well; the general and local reactions were unimportant, in contrast to the severe reactions common after parenteral immunization.

In Chile, where all attempts had failed in influencing the high typhoid fever morbidity by parenteral vaccinations, in the years 1970 and 1971 oral vaccinations were carried out in a large-scale field trial with the aim of checking this vaccine's effectivity in a country with a high typhoid fever morbidity.

In two areas, in Santiago province and the San Fernando district, 50 340 persons were immunized. The control group consisted of 54 636 persons from the same area, with the same age distribution and of the same socio-economic conditions.

As most cases of illness occur during the warm season, from October to March (Fig. 1), we vaccinated for the first time in October-November, 1970, during the highest increase in the cases of illness. After 12-15 months, the same persons were revaccinated. Vaccination was carried out with the polyvalent antityphoid vaccine "Orovaccin-Typhus", which was produced in the GDR and contains 60×10^9 typhoid and 40×10^9 paratyphoid B organisms per pill. Every proband was fed 3 pills on an empty stomach daily for three days, i.e. 9 pills with a total dose of 540×10^9 typhoid and 360×10^9 paratyphoid B killed organisms. The pills were distributed by public health personnel.

In Chile and in other developing countries with a high incidence of typhoid fever the diagnosis is generally established only clinically and a distinction is not made between typhoid or paratyphoid fever. A considerable part of the illnesses notified as typhoid fever, are in reality paratyphoid infections. The vaccine therefore must contain also the paratyphoid component.

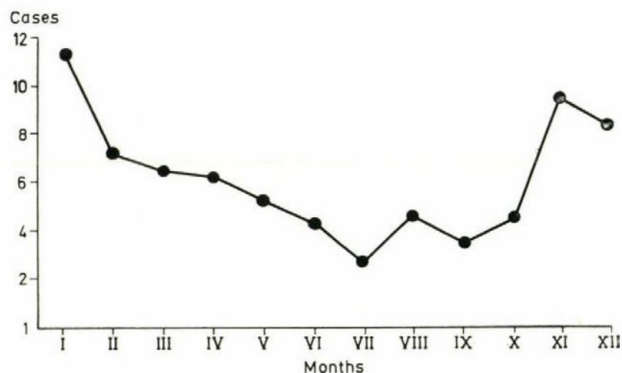


Fig. 1. Monthly distribution of typhoid fever cases 1965-1969

Table I

Incidence of typhoid fever (per 100 000 inhabitants) in Chile, in province Santiago and in San Fernando area, 1965-1969

Year	Chile	Province Santiago	San Fernando		
			rural area (vaccinated)	urban area (control)	total
1965	64.8	81.2	265.1	252.0	258.1
1966	51.5	84.4	152.1	92.1	118.2
1967	59.8	83.1	186.0	79.0	125.0
1968	75.8	142.0	279.1	144.1	207.2
1969	56.0	112.2	155.2	75.1	104.3
	59.6	100.6	207.5	128.4	162.5

According to the recent recommendations of a WHO Study Group, in the future a total dose of 4×3 pills will be administered, i.e. $4 \times 300 \times 10^9$ organisms ($4 \times 180 \times 10^9$ *S. typhi* and $4 \times 120 \times 10^9$ *S. paratyphi-B* bacteria).

The yearly typhoid fever morbidity in Chile was 59.6 per 100 000 inhabitants during the last 5 years (1965-1969), while in the province Santiago it amounted to 100.6 and in the San Fernando area to 162.5 per 100 000 inhabitants (Table I).

In the San Fernando area 60% of the population is living in urban areas and 40% in rural areas. The morbidity rates for typhoid fever in San Fernando area were higher than those of the country as a whole and also higher than of the province Santiago. In this district the rates in the rural area were higher than those in the urban area.

The analysis of the incidence of typhoid and paratyphoid fever among the age groups during the last five years in Chile shows that 89.3% of the cases occurred under the age of 35 years; most cases were observed in the age group of 10 to 14 years and amounted to 20.4% (Table II).

Table II

Distribution of typhoid and paratyphoid B cases in Chile, according to age groups, 1966–1970

Age, years	No. of cases	Per cent
0–4	274	5.0
5–9	901	16.8
10–14	1104	20.4
15–19	978	18.2
20–24	789	14.7
25–34	758	14.2
35 and older	576	10.7
Total	5380	100.0

In order to obtain reliable data on the effectiveness of the vaccine, a field trial was carried out in these age groups.

In Santiago, the age group of 10 to 20 years, and in San Fernando that of 6 to 35 years was vaccinated; the morbidity in these age groups was higher than that of children of the preschool age group and of the elder population.

Santiago. In October, 1970, a total of 39 832 schoolchildren 10 to 20 years of age from 64 schools in the south and south-eastern areas of Santiago with a morbidity rate of 115 per 100 000 inhabitants (the highest in the province of Santiago) were vaccinated. The control group consisted of 40 386 pupils of the same age group from 100 schools in the same area with similar socio-economic conditions and identical environmental sanitation. Twelve months later, 32 781 of the vaccinated children (82%) were revaccinated with the same dose.

San Fernando. In October–November, 1970, over 95% of the population aged between 6 and 35 years in the rural areas of San Fernando and the villages bordering on the community of San Fernando, were vaccinated. The total number involved was 10 508. The control group consisted of 14 250 persons of the same age in the urban sectors of San Fernando and two other communities.

Between December, 1971 and March, 1972, a total of 7850 persons was revaccinated, *i.e.* 79.5% of the population which had obtained the first vaccination in October–November, 1970.

On analyzing the effectiveness of vaccination in this district, the incidence of typhoid fever was significantly lower in the vaccinated groups than in the control groups; in addition, the vaccinations reduced also the total morbidity in this area. For the first time since 20 years was the morbidity of the rural population lower than that of the urban population. In both areas (Santiago and San Fernando) the field trial consisting of 2 immunization campaigns with an interval of 12 to 15 months, proved the high effectiveness of the oral vaccine.

An analysis carried out 12, 18 and 25 months after the first immunization and 12 months after the revaccination, showed a significant protection amounting to 75% in both areas (Tables III and IV); 75.5% protection was conferred

Table III

Incidence of typhoid fever in vaccinated and control groups in Santiago, November, 1970—December, 1972 (10 to 20 year age group)

Groups	No. of school-children	No. of cases, 12 months	Morbidity*	Protection, per cent	No. of cases, 25 months	Morbidity*	Protection, per cent
Vaccinated	39 832	6	15.1	84	16	40.1	75.5
Control	40 386	38	94.9		66	163.4	

* per 100 000

Table IV

Incidence of typhoid fever in vaccinated and control groups in San Fernando area, December, 1970—May, 1972 (6 to 35 year age-group)

Groups	No. of persons	No. of cases, 12 months	Morbidity*	Protection, per cent	No. of cases, 18 months	Morbidity*	Protection, per cent
Vaccinated	10 508	3	28.5	76.9	38	76.1	74.8
Control	14 250	31	217.5		43	301.7	

* per 100 000

by the vaccine in the field trial of Santiago and 74.8% in San Fernando. These results were important, because it was the first time that an oral killed typhoid vaccine ensured such a protection after 18 and 25 months of epidemiological observation. According to the statistical analysis, the difference in the morbidity ratio between the vaccinated and control groups was highly significant.

The vaccine was tolerated well and the general and local reactions were unimportant, in contrast to the severe reactions after parenteral immunization. In the area of Santiago, only a few persons had abdominal pains, loss

of appetite or headache 1 or 2 hr after ingesting three pills in 24 hr. In San Fernando, only 32 persons of 1000 had headache or abdominal pain.

These results show that the morbidity of typhoid fever in developing countries can successfully be reduced by oral immunization. Investigations on the duration of protection, and the optimum time of revaccination, are in progress.

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ЭКСПЕРИМЕНТАЛЬНЫЕ ИММУНОХИМИЧЕСКИЕ ИССЛЕДОВАНИЯ ТИФО-ПАРАТИФИЗНОЙ А И В ВАКЦИНЫ ИЗ ПОВЕРХНОСТНЫХ И О-АНТИГЕНОВ

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HATUNCEVA, N. V., MIRONOVA, M. V., RYAPOLOVA, R. G.: **Immunochemical examination of typhoid and paratyphoid vaccines prepared from envelope and O antigens.** In mice and rabbits Vi and O antigens of *Salmonella typhi* and envelope and O antigens of *Salmonella paratyphi-A* and *Salmonella paratyphi-B* produced immunity after oral administration. The combined tabletted typhoid-paratyphoid vaccine prepared on the basis of the experiments was highly effective for the immunization of laboratory animals.

Резюме. Были проведены экспериментальные исследования, посвященные оральной иммунизации против тифо — паратифозных инфекций. Было установлено, что Ви и О — брюшнотифозные антигены, так же как поверхностные и О — паратифозные А и В антигены способны при пероральном введении их белым мышам и кроликам вызывать поствакцинальный иммунитет, напряженность и длительность которого зависит от способа извлечения антигена, кратности иммунизации, заражающей дозы и других факторов. В результате проведенных исследований была получена ассоциированная тифо-паратифозная А и В вакцина в таблетках, состоящая из поверхностных и О — антигенов. В опытах на животных была выявлена высокая активность препарата, но окончательная оценка может быть сделана только после изучения его эффективности и реактогенности в эпидемиологических условиях.

За последнее десятилетие вопросам энтеральной иммунизации против кишечных инфекций придается большое значение. Многочисленные работы исследователей указывают на эффективность и перспективность перорального метода вакцинации [1—6].

В. Л. Троицким, В. Д. Геккер, Н. И. Ковалевой, Б. Б. Першиным, А. Н. Мешаловой и др. было обосновано применение в качестве вакцинных препаратов для энтеральной иммунизации против кишечных инфекций антигенных комплексов, преимуществом которых перед корпускулярными вакцинами является более быстрая всасываемость желудочнокишечным трактом и отсутствие инактивирующего воздействия на них желудочного сока.

С 1962 года в ИЭМ им. Гамалеи АМН СССР велись исследования по изучению некоторых вопросов энтеральной иммунизации против кишечных инфекций. Одной из задач этих исследований являлось разработать химическую энтеральную вакцину против брюшного тифа и паратифов А и В, полагая, что для создания поствакцинального иммунитета в препарат должны входить антигенные комплексы, состоящие из поверхностных, О и Н — антигенов.

Антигены выделяли из культур брюшного тифа штамм Ty₂—4446, паратифа А штамм 50503 и паратифа В штаммы 42 и 50602, выращенных в 500 литровых реакторах в условиях глубинного культивирования на полусинтетической среде.

Ви — брюшнотифозные и поверхностные паратифозные А и В антигены извлекали из микробной массы водно-солевой экстракцией с последующим осаждением спиртом по методу Ключевой [7]. О-антигены получали по методу Буавена [8].

Полученные антигенные комплексы были охарактеризованы по химическим показателям и антигенному строению, а также изучены их токсичность, серологические и иммуногенные свойства.

Результаты сравнительного изучения препаратов, представленные в таблице I, показывают, что по химическому составу антигены различались

Таблица I

Иммунохимическая характеристика антигенных комплексов

Культура	Антиген	Общий азот	Белок	Общий фосфор	Нуклеиновые к-ты	Сахара редуц.	Гексозамин	Липиды	Токсичность LD ₅₀ в мг	Титр преципитации	Иммуногенная активность ЕД ₅₀ в мг
	в % на абсолютное сухое обеззоленное вещество										
<i>S. typhi</i>	Ви О	9,2 3,4	61,7 20,5	1,7 2,1	18,2 1,8	12,4 40,6	1,2 3,8	10,4 12,0	1,94 0,14	1:128 000 1:256 000	0,0002 0,00004
<i>S. paratyphi-A</i>	Поверхностный О	8,3 2,5	49,3 16,7	2,0 1,3	23,1 3,8	26,5 41,0	0,9 3,3	7,9 12,2	1,8 0,20	1:128 000 1:256 000	0,0025 0,0020
<i>S. paratyphi-B</i>	Поверхностный О	10,1 3,0	62,9 19,8	2,5 1,2	20,1 3,6	9,5 42,4	2,0 3,9	10,2 11,6	1,23 0,18	1:128 000 1:256 000	0,0013 0,0011

между собой. Наибольшее количество белка наблюдалось в Ви и поверхностных антигенах (49,3—62,9%) и значительно меньшее в О-антигенах (16,7—20,5%). Существенные различия были установлены в содержании нуклеиновых кислот (18,2—23,1% в поверхностных и 1,8—3,6% в О-антигенах). Достоверные различия наблюдались также в количестве редуцирующих сахаров и гексозамина.

Ви и поверхностные антигены являлись слаботоксичными препаратами (LD₅₀ их равнялась 1,23—1,99 мг) по сравнению с О-антигенами, LD₅₀ которых составляла 0,14—0,20 мг. Все препараты обладали выраженной антигенной и иммуногенной активностью (табл. I).

Исследование антигенного состава препаратов методом иммуноэлектрофореза выявило сложное их строение. Брюшнотифозные Ви и поверхностные паратифозные А и В антигены состояли из нескольких компонентов, обладающих различной электрофоретической подвижностью, а именно: из собственно поверхностного антигена, комплекса из поверхностных и О-антигенов и Н-антигена, присутствие которого указывает на недеградированность препаратов. О-антигены по Буавену образовывали по 2 линии преципитации, форма и расположение которых являлись типичными для О-соматических антигенов.

Проведенные нами экспериментальные исследования установили, что Ви и О брюшнотифозные антигены, а также поверхностные и О-паратифозные А и В антигены способны при пероральном введении их белым мышам и кроликам вызывать поствакцинальный иммунитет, напряженность и длительность которого зависела от способа извлечения антигена, кратности иммунизации, заражающей дозы и других факторов.

В результате проведенных исследований первоначально была создана энтеральная вакцина из Ви и О-брюшнотифозных антигенов в таблетках, предназначенная для специфической профилактики населения.

В эксперименте была установлена высокая эффективность препарата [9, 10]. Испытание вакцины на 500 добровольцах показало, что препарат практически безвреден [11].

В связи с тем, что в литературе имеются ссылки на недостаточную эффективность паратифозных компонентов существующих вакцин, наша дальнейшая работа была направлена на разработку ассоциированного препарата — энтеральной химической тифо-паратифозной А и В вакцины в таблетках.

Изучение иммуногенной активности моноантигенов показало, что все испытанные препараты были способны при однократном пероральном введении вызывать поствакцинальный иммунитет. Величина ED_{50} их составляла 0,25—2,0 мг (табл. II). По сравнению с подкожным методом введения — для эффективной пероральной иммунизации требовались примерно в 1000 раз большие дозы антигенов, что согласуется с работами других исследователей [2, 12—14].

В многочисленных опытах было установлено, что сочетание поверхностных и О-антигенов усиливает иммуногенную активность препарата по сравнению с антигенами, введенными раздельно (рис. 1). Особенно наглядно это было продемонстрировано при жестком контроле заражения ($8-10LD_{50}$). Так, поверхностные антигены обуславливали 35—55% выживаемости мышей; О-антигены — 25—35%; мыши, иммунизированные смесью из этих антигенов выживали, в 70—90%.

Анализируя полученные данные, нам кажется вполне оправданным включение в вакцинные препараты строго дозированных антигенных комплексов, состоящих из поверхностных и О-антигенов.

Таблица II
Иммуногенная активность антигенов при однократном введении

Культура	Антиген	Способ введения	Величина ЕД ₅₀ в мг и пределы колебаний
<i>S. typhi</i> Ту ₂ и 4446	Ви	перорально	0,25 (0,17 ÷ 0,35)
		подкожно	0,00002 (0,00001 ÷ 0,00003)
	О	перорально	0,62 (0,41 ÷ 0,83)
		подкожно	0,00004 (0,00002 ÷ 0,00006)
<i>S. paratyphi-A</i> и 50503	Поверхностный	перорально	1,7 (1,2 ÷ 2,1)
		подкожно	0,0025 (0,0015 ÷ 0,0038)
	О	перорально	2,0 (1,7 ÷ 2,3)
		подкожно	0,0020 (0,0014 ÷ 0,0029)
<i>S. paratyphi-B</i>	Поверхностный	перорально	1,1 (0,8 ÷ 1,6)
		подкожно	0,0013 (0,0007 ÷ 0,0019)
N. 42 и 50602	О	перорально	0,9 (0,7 ÷ 1,5)
		подкожно	0,0011 (0,0006 ÷ 0,0017)

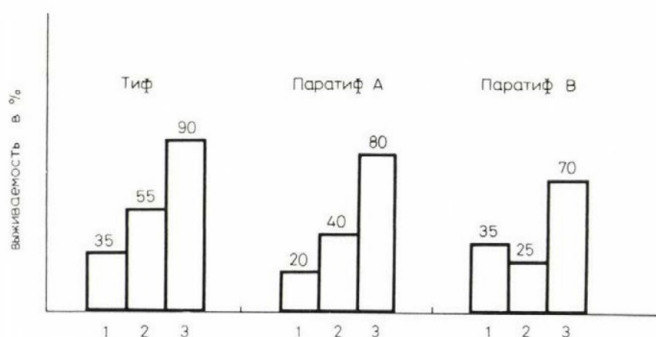


Рис. 1. Иммуногенные свойства антигенных комплексов при пероральном методе введения. 1 — О-антиген, 2 — поверхностный антиген (Ви), 3 — поверхностный + О-антигены (заражение 8—10 ЛД₅₀ тест-культур)

Особое внимание было уделено вопросу о кратности иммунизации. Трехкратное введение антигенов повышало активность препаратов по сравнению с однократным введением в 2—3 раза. Наиболее эффективной оказалась схема дробной иммунизации: два цикла по 3 дня подряд с интервалом в 10—15 дней, позволяющая вводить в организм массивные дозы антигенов.

После проведения ряда исследований, касающихся разработки оптимальных доз антигенов, напряженности и длительности иммунитета, была приготовлена ассоциированная тифо-паратифозная А и В вакцина в таблетках для перорального применения. Каждая таблетка содержала 15 мг антигенов (3,5 мг Ви и 1,5 мг О-брюшнотифозных антигенов и по 2,5 мг поверхностных и О-паратифозных А и В антигенов).

Изучение эффективности полученного препарата показало, что наиболее высокий уровень иммунитета наблюдается к 10 дню после курса иммунизации (75—100% выживаемости животных) и снижается к 60 дню. Через

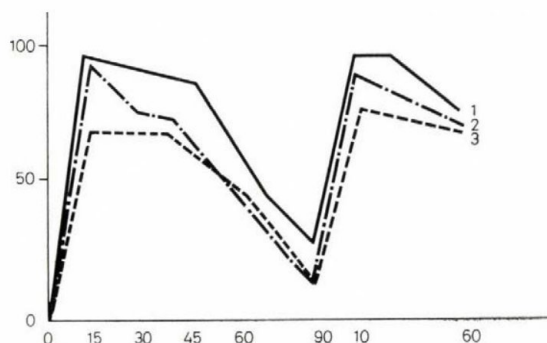


Рис. 2. Эффективность энтеральной ТАВ — вакцины. Стрелкой указана ревакцинация, по оси абсцисс — сроки заражения (в днях), по оси ординат — выживаемость в %. Компоненты вакцины: 1 — тифозный; 2 — паратифозный А; 3 — паратифозный В

3 месяца выживаемость мышей не превышает 17—20%; ревакцинация, проведенная на спаде иммунитета, повышала уровень и удлиняла продолжительность иммунитета в отношении каждого компонента вакцины (рис. 2).

Особого внимания заслуживает вопрос о конкуренции компонентов вакцины. Как известно, данные литературы свидетельствуют об отсутствии конкурирующего действия компонентов подкожных ассоциированных химических вакцин. Наши наблюдения дают повод полагать, что и при энтеральном методе введения конкурирующего действия между компонентами вакцины не наблюдается. Как следует из данных таблицы III, иммуногенная активность ассоциированного препарата не отличалась от моновакцин.

Из многочисленных работ известно, что пероральная иммунизация против кишечных инфекций вызывает в организме более слабое образование антител, чем подкожная [3]. Наши исследования подтвердили это положение; сыворотки перорально иммунизированных белых мышей и кроликов, испытанные в реакции агглютинации и РПГА, имели довольно низкие титры антител, особенно в отношении поверхностных антигенов. Отмечалось также отсутствие корреляции между антигенными свойствами вакцины и ее иммуногенной активностью.

Таблица III

Иммуногенные свойства ассоциированной ТАВ-вакцины по сравнению с моновакцинами

Компонент	Заражение культурой (4–5 LD ₅₀)	% $\pm$ mр выживаемости мышей	
		ТАВ-вакцина	моновакцина
Тифозный	<i>S. typhi</i> 4446	90 $\pm$ 3,2	85 $\pm$ 2,9
Паратифозный А	<i>S. paratyphi-A</i> 50503	100 $\pm$ 3,1	90 $\pm$ 3,2
Паратифозный В	<i>S. paratyphi-B</i> 50602	70 $\pm$ 2,8	70 $\pm$ 2,8

Таблица IV

Превентивные свойства антисывороток

Антисыворотка	Величина РД ₅₀ в мл и пределы ее колебаний	
	До иммунизации	После иммунизации
Тифозная	0,19 (0,14 $\div$ 0,29)	0,0016 (0,0012 $\div$ 0,0022)
Паратифозная А	0,19 (0,14 $\div$ 0,29)	0,008 (0,004 $\div$ 0,0124)
Паратифозная В	0,19 (0,14 $\div$ 0,29)	0,004 (0,002 $\div$ 0,007)

Тем не менее сыворотки мышей, иммунизированных энтеральной тифо-паратифозной вакциной, обладали выраженными превентивными свойствами, как в отношении культур брюшного тифа, так и паратифов А и В. Как свидетельствуют результаты опытов, представленные в таблице 4, величина РД₅₀ сывороток до иммунизации равнялась 0,19 мл. После иммунизации (через 7 дней) РД₅₀ антисывороток составляла: против тифа — 0,0016 мл, против паратифа А и В — 0,004–0,008 мл соответственно.

Проведенные экспериментальные исследования позволяют сделать следующее заключение:

Ассоциированная тифо-паратифозная А и В вакцина в таблетках, состоящая из поверхностных и О-антигенов, способна создавать при пероральном методе введения поствакцинальный иммунитет достаточной напряженности.

Окончательная оценка препарата может быть сделана только после изучения его эффективности и реактогенности в эпидемиологических условиях.

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GENERAL IMMUNITY REACTIONS AFTER ORAL ADMINISTRATION OF INACTIVATED TYPHOID VACCINES IN MAN

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Summary. Results concerning the general immunity reactions (production of the specific agglutinating antibodies O, Vi, H; changes in the specific serum bactericidal activity and the specific phagocytizing capacity of leucocytes) after oral administration to humans of inactivated typhoid vaccines are presented. Vaccines manufactured by different technology and/or the same vaccine given in different doses and immunization schedules were compared. The specificity of immunity reactions was also tested. The results allowed to conclude that (i) oral administration of inactivated typhoid whole-cell vaccines may lead to a general immunity reaction manifested by a measurable and often even significant increase in the specific agglutinating antibodies, a significant increase in the specific phagocytizing capacity of leucocytes, and a significant rise in specific serum bactericidal activity; (ii) intensity of the general immunity reactions was dependent on the manufacturing technology as well as on dosage and immunizing schedule. On the basis of the results, a suitable dose of vaccine and a suitable immunizing schedule are recommended.

This paper reports on a study of the immunogenicity of oral typhoid vaccines administered to human volunteers, on the basis of general immunity reactions. For this purpose, we observed the production of specific agglutinating antibodies (O, Vi, H), the specific serum bactericidal activity (BA) expressed by means of the bactericidal index (BI), in various periods after immunization. In some cases, the phagocytizing capacity of leucocytes expressed by means of the phagocytosis index (PI) was also studied.

The methods applied have been described earlier [1]. After demonstrating the ability of oral typhoid vaccines to provoke marked immunity reactions of a general character, we have studied the intensity of immunity reactions after administration of vaccines manufactured by different technology and/or vaccines administered in different doses and immunization schedules.

Besides, the specificity of immunity reactions was checked by using a vaccine from *Plesiomonas shigelloides* and a vaccine from *Shigella dysenteriae* 8. All typhoid vaccines used were manufactured from the standard strain *Salmonella typhi* Ty 2.

First, we studied the immunogenicity of a vaccine manufactured from acetone-killed and dried bacterial biomass, obtained by surface cultivation on nutrient agar and administered in the form of a bacterial suspension in saline, in comparison with the same type of vaccine, administered in pills with an enterosolvent coating. Both vaccines were administered in a total dose of

100 mg corresponding to 300×10^8 germs, divided into 2 portions given on 2 consecutive days.

Next, the qualities of a vaccine prepared from surface culture were compared with those of a vaccine made from continuous culture in nutrient broth, both of them being administered in the form of coated pills in a total dose of 100 mg on a single occasion.

Both these vaccines caused a significant rise in O and Vi agglutinating antibodies 1–4 weeks after vaccination (Tables I and II) and a significant rise in the specific serum BI value for a period of 6 months. The latter reached significantly higher levels after vaccination with pills.

Table I

Specific agglutinating antibodies in serum of persons before and 7 days after administration of typhoid enterovaccine in coated pills

Type of antibody	Titre	Before vaccination		7 days after vaccination		Significance of difference between groups
		absolute	per cent	absolute	per cent	
O	0	3	11.5	—	—	$P < 0.01$
	10	4	15.4	—	—	
	20	8	30.8	3	11.5	
	40	8	30.8	10	38.5	
	80	3	11.5	10	38.5	
	160	—	—	3	11.5	
	Total	26	100.0	26	100.0	
Vi	0	19	73.1	12	76.2	$P < 0.05$
	2	5	19.2	2	7.7	
	4	2	7.7	10	38.5	
	8	—	—	1	3.8	
	16	—	—	1	3.8	
	Total	26	100.0	26	100.0	

Administration of the control vaccine made from *P. shigelloides* caused no immune reactions specific of *S. typhi* (Fig. 1).

Likewise, a rise in the specific PI values was registered 3–28 days after vaccination (Table III). After the administration of vaccines in the form of pills from a continuous or surface culture in a total dose of 100 mg on a single occasion, a statistically non-significant rise in O agglutinating antibodies occurred 1–3 weeks after vaccination in 42% of the subjects. Similarly, an increase in specific serum BI within 1 week to 5 months after vaccination was found in 44% of the subjects and in significantly more after the administration

Table II

Specific agglutinating antibodies in serum of persons before and 7 days after administration of typhoid enterovaccine in the form of bacterial suspension

Type of antibody	Titre	Before vaccination		7 days after vaccination		Significance of difference between groups
		absolute	per cent	absolute	per cent	
O	0	2	8.3	—	—	$P < 0.005$
	10	1	4.2	—	—	
	20	9	37.5	1	4.2	
	40	9	37.5	6	25.0	
	80	3	12.5	9	37.5	
	160	—	—	5	20.8	
	320	—	—	3	12.5	
	Total	24	100.0	24	100.0	
Vi	0	15	62.5	8	33.3	$P < 0.05$
	2	3	12.5	—	—	
	4	6	25.0	10	20.8	
	8	—	—	5	4.2	
	16	—	—	1	—	
	Total	24	100.0	24	100.0	

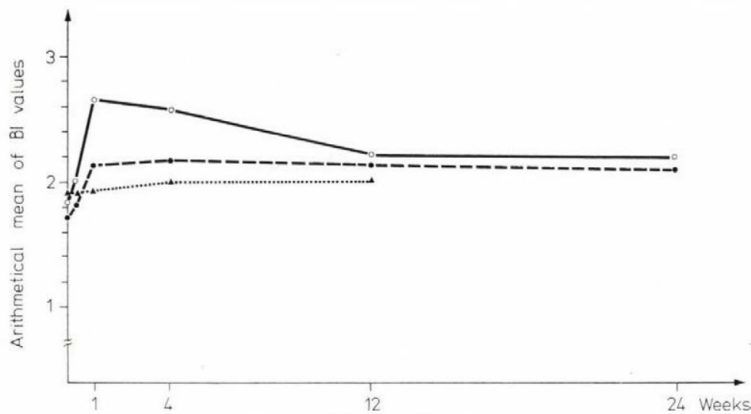


Fig. 1. Serum BI values against *S. typhi* after the administration of *S. typhi* and *P. shigelloides* enterovaccines. — Typhoid enterovaccine in pills; - - - - typhoid enterovaccine in bacterial suspension; enterovaccine from *P. shigelloides*

of the surface culture vaccine. After the administration of control vaccine from *S. dysenteriae* 8, no *S. typhi* specific immunity developed (Fig. 2).

A significant increase in the specific PI values was also registered 3–21 days after the vaccination (Table IV). Administration of the vaccine in a single dose was not sufficient for eliciting a proper immunity reaction.

Table III*PI values in blood of persons after administration of typhoid enterovaccines*

Group	No. of subjects	Arithmetical mean and standard error of mean of PI values			
		before vaccination	3 days	7 days	28 days
			after vaccination		
I	26	0.000	0.032 ± 0.008	0.026 ± 0.007	0.004 ± 0.002
II	24	0.002 ± 0.001	0.045 ± 0.018	0.019 ± 0.007	0.021 ± 0.005

Group I. Typhoid enterovaccine in pills

II. Typhoid enterovaccine in bacterial suspension

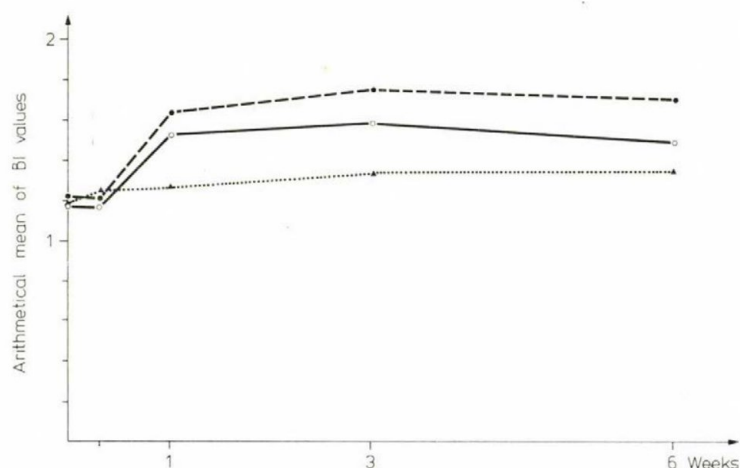


Fig. 2. Serum BI values against *S. typhi* after the administration of typhoid and *S. dysenteriae* 8 enterovaccines. — Typhoid enterovaccine from continuous culture administered in a single 100 mg dose; - - - - typhoid enterovaccine from surface culture administered in a single 100 mg dose; enterovaccine from *S. dysenteriae* 8.

Table IV*PI values in blood of persons after administration of typhoid enterovaccines*

Group	No. of subjects	Arithmetical mean and standard error of mean of PI values			
		before vaccination	3 days	7 days	28 days
			after vaccination		
I	10	0.004 ± 0.002	0.028 ± 0.012	0.047 ± 0.017	0.027 ± 0.009
II	12	0.000	0.025 ± 0.012	0.022 ± 0.010	0.012 ± 0.006

Group I. Typhoid enterovaccine from continuous culture

II. Typhoid enterovaccine from surface culture

Data on the methods used and the pertaining results have been presented elsewhere in more detail [2].

Subsequently, the effects of different vaccine doses and different immunization schedules have been compared. In these experiments the same type of acetone-killed and dried vaccine from a continuous culture was used. Three series of experiments were carried out.

1. Comparison of a 100 mg dose administered in 3 fractions on 3 consecutive days, with a 200 mg dose administered in 2 portions on 2 consecutive days.

2. Comparison of a 100 mg dose administered in 3 fractions on 3 consecutive days, with the same dose administered in 3 fractions every other day, with a 200 mg dose administered in 3 portions on 3 consecutive days, and with a 50 mg dose administered in 4 fractions every other day.

3. Comparison of a 100 mg dose administered in 3 fractions on 3 consecutive days, with the same dose administered in 5 portions on 5 consecutive days, and with a 200 mg dose administered in 5 fractions on 5 consecutive days.

The results were as follows. In all these experiments, a transient increase in O agglutinating antibodies, which in some cases proved statistically significant, was registered. The increase in Vi agglutinating antibodies was weak or lacking. There was no production of H antibodies in any case. Changes in the specific serum BA were considered the main criterion of the immunogenicity of vaccines.

There was no significant difference in intensity of immunity after the 100 mg dose administered in 3 fractions on 3 consecutive days, and the 200 mg dose administered in 2 fractions on 2 consecutive days.

No significant difference was observed between the total 100 mg and 200 mg doses administered in 3 fractions on 3 consecutive days. On the other hand, a weak immunity reaction was noted after a 100 mg total dose given in 3 fractions every other day, and the immunity response was insufficient after the administration of 50 mg of the vaccine (Fig. 3).

No significant difference in intensity of the immunity reaction was found after the 100 mg dose administered in 3 fractions on 3 consecutive days, and the same dose administered in 5 fractions on 5 consecutive days. Likewise, there was no difference between the 100 mg dose administered in 5 fractions on 5 consecutive days and the 200 mg dose administered in the same way (Fig. 4). These results have been presented elsewhere in more detail [2, 3].

When evaluating the immunogenicity of typhoid vaccines, mainly the changes in the specific BA of serum are taken into consideration, for the following reasons.

1. The specific serum BI is significantly higher in persons with a history of typhoid than in those without typhoid in their history. The high level of specific serum BA persists for a long period and it seems to be in correlation with a prolonged immunity against typhoid (Table V).

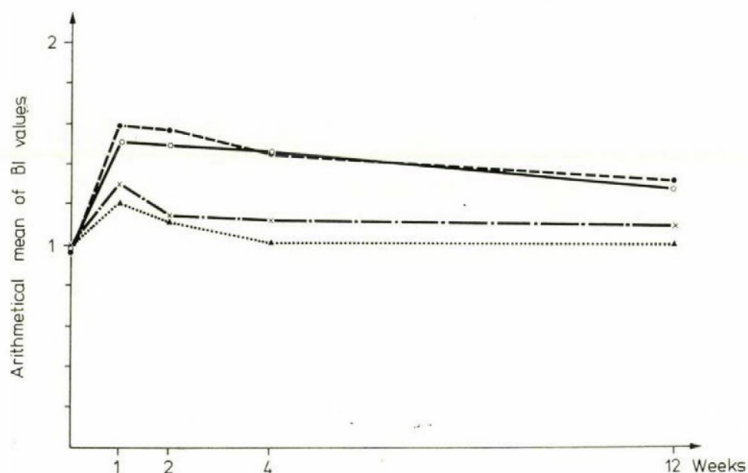


Fig. 3. Serum BI values against *S. typhi* after the administration of typhoid enterovaccine in various doses and various immunization schedules. — Enterovaccine in 100 mg dose, administered in 3 fractions on 3 consecutive days; - - - - - enterovaccine in 100 mg dose, administered in 3 fractions every other day; - . - . - enterovaccine in 200 mg dose, administered in 3 fractions on 3 consecutive days; enterovaccine in 50 mg dose, administered in 4 fractions every other day

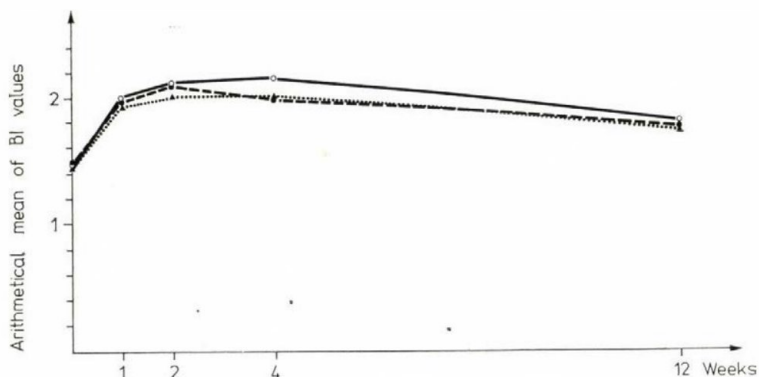


Fig. 4. Serum BI values against *S. typhi* after the administration of typhoid enterovaccine in various doses and various immunization schedules. — Enterovaccine in 100 mg dose, administered in 3 fractions on 3 consecutive days; — — — — — enterovaccine in 100 mg dose, administered in 5 fractions on 5 consecutive days; enterovaccine in 200 mg dose, administered in 5 fractions on 5 consecutive days

2. The increase in the specific serum BI values in man and experimental animals was significantly higher after the parenteral administration of the standard WHO K vaccine than after the standard L vaccine (Tables VI and VII). This result agrees with those achieved in controlled field trials in which the K vaccine was found to afford a significantly higher protection than did the L vaccine.

Table V

Serum BI values against S. typhi in persons with typhoid in the history as compared with those without typhoid in the history

Group examined	No. of subjects	Arithmetical mean and standard error of mean of BI values	Significance of difference
Persons with typhoid history	71	3.07 ± 0.07	$P < 0.001$
Persons without typhoid history	74	$1.97 - 0.13$	

Table VI

Serum BI values against S. typhi before and after vaccination with typhoid K and L vaccines in doses of 1000 million bacteria each (serum dilution 1 : 25)

Vaccine	No. of subjects	Arithmetical mean and standard error of mean of BI values	
		Before vaccination	3 weeks after vaccination
K	27	0.18 ± 0.05	1.23 ± 0.04
L	25	0.18 ± 0.05	0.93 ± 0.08

Table VII

Values for serum BI against S. typhi in rabbits immunized with K and L typhoid vaccines (experiment 2)

Vaccine	Number of animals	Arithmetical mean and standard error of mean of BI values				
		Before immunization	After the 1st dose of vaccine		After the 2nd dose of vaccine	
			7 days	14 days	7 days	28 days
K	15	0	0.57 ± 0.13	0.55 ± 0.1	0.11 ± 0.06	0.51 ± 0.1
L	15	0	0.04 ± 0.02	0.08 ± 0.04	0.06 ± 0.02	0.11 ± 0.05

Summing up, oral administration of inactivated typhoid whole-cell vaccines may lead to a general immunity manifesting itself with an often statistically significant increase in specific agglutinating antibodies, a significant increase in the specific phagocytizing capacity of leucocytes, and especially with a significant increase in the specific serum BA. The intensity of these reactions depends on the manufacturing technology of the vaccine as well as on its dosage and immunizing schedule.

The additional local immunity developing in the gastrointestinal tract may also be important in the protection against typhoid.

On the basis of our results, we recommend an amount of 100 mg dried bacterial biomass, corresponding to 300×10^9 germs as a total dose of oral typhoid vaccine for adults, and to administer the vaccine in 3 fractions on 3 consecutive days. Still, these results will have to be controlled in field trials in order to prove the real protective effect of this method of vaccination.

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СРАВНИТЕЛЬНАЯ ОЦЕНКА МЕХАНИЗМА ИММУНИТЕТА ПРИ ЭНТЕРАЛЬНОЙ И ПОДКОЖНОЙ ИММУНИЗАЦИИ ТИФО-ПАРАТИФОЗНЫМИ ВАКЦИНАМИ

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KRASNOPROSHINA, L. I.: **Comparison of oral and subcutaneous immunization against typhoid-paratyphoid fever.** The distribution of ^{14}C -labelled *Salmonella typhi* Boivin antigen in the organs of animals was studied. One hr after oral administration the antigen was shown in the mesenterial lymph nodes and in the liver of mice; after 24 hr the antigen accumulated in the spleen. Subcutaneous injection resulted in the appearance of the antigen in the blood and spleen (1 hr), then in the liver and mesenterial lymph nodes (24 hr). In rabbits the antigen was present mainly in the lymphatic tissues of the appendix and intestinal tract and in the mesenterial lymph nodes; it appeared in low amounts in the organs. After oral immunization the spleen, liver and lymph nodes contained one hundred times less antigen than after subcutaneous injection. The antigen content of the lymphoid cells run parallel with the amount of antigen in antibody-synthesizing cells. In rabbits antibody synthesizing cells appeared in the small intestine, appendix and mesenterial lymph nodes considerably earlier after oral than after subcutaneous immunization. The results indicate that oral vaccination yields a more rapid immunity than subcutaneous injection.

Резюме: Изучено распределение антигена в органах животных после энтерального и подкожного введения антигена Буавена *S. typhi*, содержащего радиоактивную группу (C^{14}). Установлено, что через 1 час после пероральной иммунизации мышей радиоактивный антиген проникает через слизистую оболочку кишечника и обнаруживается в мезентериальных лимфатических узлах и печени. В селезенке перорально иммунизированных мышей радиоактивный антиген накапливается через 24 часа. После подкожного введения радиоактивный антиген вначале обнаруживается в селезенке и крови (через 1 час), а затем в печени и мезентериальных узлах (через 24 часа). Аналогичные явления были отмечены при введении радиоактивного антигена в полость тонкой кишки кроликов. При этом большое количество радиоактивной метки присоединялось к ткани аппендикса, кишечника, мезентериальных узлов. Подкожная иммунизация кроликов сопровождалась более поздним и менее значительным накоплением антигена в этих органах. Более всего антигена было найдено в регионарном лимфатическом узле кроликов после подкожного введения. Было вычислено, что в селезенку, лимфатические узлы, печень перорально иммунизированных животных попадает в 100 раз меньше антигена, чем в эти органы после подкожного введения. Выявлена прямая зависимость между содержанием в лимфоидных органах (селезенке, лимфатических узлах, ткани кишки) радиоактивного антигена и клеток, синтезирующих антитела (определяемых по методу Jerne—Nordin). Показано, что энтеральная иммунизация кроликов тифо-паратифозными вакцинами сопровождается более ранним появлением в ткани тонкой кишки, аппендикса, мезентериальных узлах клеток, синтезирующих антитела, по сравнению с подкожной иммунизацией. Полученные данные позволяют рекомендовать метод энтеральной иммунизации против кишечных инфекций, как обеспечивающий более быстрое развитие иммунитета на пути «входных ворот» инфекции.

Пероральный метод иммунизации обладает рядом преимуществ по сравнению с наиболее применяемым в настоящее время подкожным. К ним относятся: простота введения, безболезненность, отсутствие тяжелых по-

бочных реакций, возможность в короткий срок провакцинировать большие группы населения. Несмотря на длительную историю вопроса и большое число исследований, этот метод не внедрён в практику профилактики тифо-паратифозных заболеваний. Одной из причин являлось недостаточное знание механизма распространения антигена из желудочно-кишечного тракта, последовательности возникновения иммунологических реакций в ответ на пероральное введение вакцин. В связи с этим целью настоящей работы было изучение механизма иммунитета при пероральной вакцинации и его особенности по сравнению с подкожным путём, разработки следующих вопросов:

1) изучение судьбы антигена, введённого перорально, возможность его прохождения через стенку кишечника и накопление в различных органах;

2) изучение корреляции между содержанием антигена и степенью развития иммунологических реакций в антителиобразующих органах перорально иммунизированных животных по сравнению с иммунизированными подкожно;

3) изучение факторов, обуславливающих появление местных и общих специфических иммунологических реакций, после пероральной иммунизации в сравнении с подкожной.

Судьба антигена, введённого в желудочно-кишечный тракт, была исследована с помощью применения меченного радиоактивным углеродом (C^{14}) антигена Буавена брюшнотифозного микроба. Меченный антиген был приготовлен в лаборатории изотопов Горьковского ИЭМ Н. И. Герасимовым и В. М. Лавровской из культуры брюшнотифозной палочки, выращенной на среде с добавлением радиоактивной соли уксусно-кислого натрия. Антиген обладал удельной радиоактивностью порядка 500 000 имп/мин на 1 мг вещества.

Распространение радиоактивного антигена и быстроту его прохождения через стенку кишечника изучали на двух экспериментальных моделях — пероральной вакцинации мышей и внутрикишечной вакцинации кроликов в сравнении с подкожной иммунизацией.

Мышам перорально с помощью специальной канюли вводили по 3 мг радиоактивного антигена, подкожно — по 0,15 мг. В различные промежутки времени после вакцинации мышей вскрывали, готовили гомогенаты органов, наносили их на специальные мишени для дальнейшего подсчета радиоактивности, которое проводили на установке «Волна».

Было установлено, что через 1 час после пероральной иммунизации мышей радиоактивный антиген в наибольшем количестве находился в ткани кишечника. В это же время высокая радиоактивность была обнаружена в ткани брыжеечных лимфатических узлов и печени, и только следы радиоактивного антигена присутствовали в ткани селезёнки и крови. Через 24 часа содержание антигена снижалось в ткани кишечника и брыжеечных

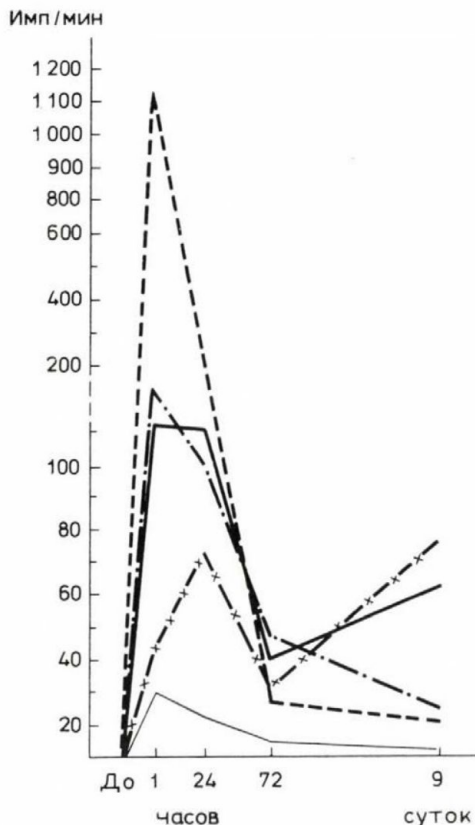


Рис. 1. Распределение меченого антигена Буавена в органах мышей после перорального введения антигена в дозе 3 мг на мыш (в импульсах в минуту на 100 мг ткани органа). Условное обозначение: — — в ткани тонкой кишки, — · — в брыжеечных лимфатических узлах, — в печени, — x — в селезенке, — в крови

узлов, повышалось в селезенке. Спустя 3 суток после пероральной иммунизации содержание антигена уменьшалось во всех исследованных органах (Рис. 1).

После подкожной иммунизации мышей через 1 час наибольшее количество радиоактивного антигена было найдено в ткани селезенки и в крови, меньше — в ткани печени, а в брыжеечных лимфатических узлах и стенке кишечника антиген почти не обнаруживался (Рис. 2). Спустя 24 часа содержание антигена у подкожно иммунизированных мышей в ткани селезенки уменьшалось, а в печени, брыжеечных узлах и ткани кишечника — увеличивалось. Через 3 суток содержание антигена снижалось во всех изученных органах.

Из результатов этих опытов можно заключить, что введенный в ротовую полость антиген быстро (в течение 1 часа) проходит через стенку желудка —

кишечного тракта и достигает органов, имеющих большое значение в развитии иммунного ответа — мезентериальных узлов, а позднее — селезёнки. В отличие от пероральной иммунизации после подкожного введения содержание антигена сначала увеличивается в селезёнке, а затем в мезентериальных узлах и стенке кишечника. На 3 и 9 дни содержание антигена существенно не различалось у перорально и подкожно иммунизированных мышей.

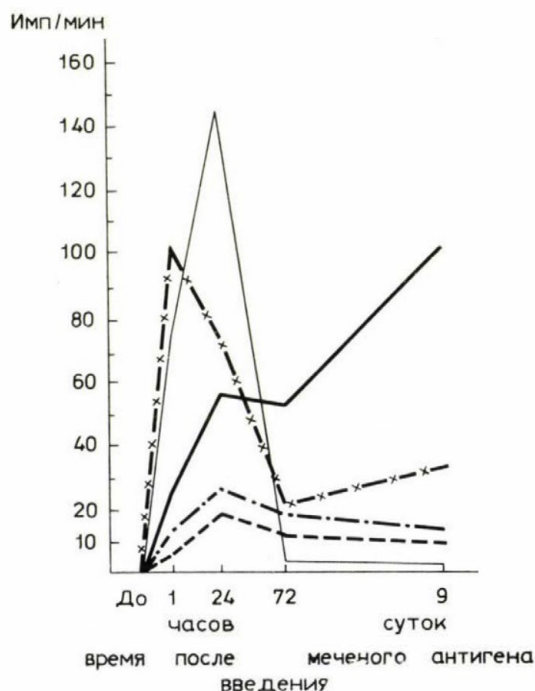


Рис. 2. Распределение меченого антигена Буавена в органах мышей после подкожного введения антигена в дозе 0,15 мг на мыш (в импульсах в минуту на 100 мг ткани органа). Условное обозначение: — · — в ткани тонкой кишки, — · · — в брыжеечных лимфатических узлах, — в печени, — x — в селезенке, — в крови.

Сходные результаты были получены в опытах внутрикишечной и подкожной иммунизации кроликов. Кроликам вводили непосредственно в полость тонкой кишки (оперативным путём) 2 мг на кг веса животного антигена Буавена, а контрольным кроликам при подкожной иммунизации — 0,4 мг на кг веса.

Через 6 часов после внутрикишечной иммунизации (до этого срока определение антигена в органах не проводили) радиоактивный антиген в большом количестве обнаруживался в ткани кишечника, аппендикса и мезентериальных лимфатических узлов. Спустя одни сутки после внутрикишечной иммунизации отмечали увеличение радиоактивности в ткани

печени и селезёнки, но содержание антигена в ткани кишечника было в этот срок значительно выше. Накопление антигена в печени, селезёнке и отдалённых лимфатических узлах продолжало повышаться до 3 суток после внутрикишечной иммунизации. На 14 день во всех исследованных органах содержание антигена снижалось.

При подкожной иммунизации кроликов через 6 часов наибольшее количество антигена обнаруживалось в ткани регионарного лимфатического узла, на втором месте находилась ткань селезёнки, на третьем — печени. Радиоактивность мезентериальных узлов, ткани аппендикса и кишечника была очень низкой. Спустя сутки, содержание радиоактивной метки увеличивалось в ткани брыжеечных лимфатических узлов, отдалённых (подколенных) лимфоузлов, стенке аппендикса, но оставалось на низком уровне в ткани тонкой кишки.

Таким образом, основная закономерность, отмеченная в опытах внутрикишечной иммунизации кроликов, заключалась в том, что введение радиоактивного антигена в полость кишечника сопровождается его значительной адсорбцией тканями желудочно-кишечного тракта — стенкой кишечника, аппендикса, а также мезентериальными узлами; подкожная иммунизация обуславливает более позднее и менее значительное накопление антигена в этих органах.

В связи с тем, что нахождение в ткани радиоактивного вещества ещё не является прямым доказательством присутствия антигена, а может быть отщепившейся от антигена радиоактивной меткой, в работе был применён ряд контролей. Во-первых, прочность связи антигена с изотопной группой была установлена путём использования специфического иммуносорбента, содержащего О-брюшнотифозные антитела. Иммуносорбент был приготовлен Т. С. Котовой и В. А. Ляшенко в лаборатории иммунохимии института имени Мечникова. При смешивании гомогенатов органов иммунизированных животных с иммуносорбентом 80—90% радиоактивной метки присоединялось к сорбенту, а так как к О-антителам мог присоединиться только О-антиген, то это свидетельствовало, что в организме иммунизированных животных радиоактивная группа была прочно связана со специфическим антигеном. Во-вторых, одновременно с определением антигена по радиоактивности гомогенатов органов, в тех же самых гомогенатах определяли содержание О-антигена по реакции торможения гемагглютинации. Оба метода показали сходные результаты, но метод радиоактивной индикации оказался несколько более чувствительным. В-третьих, характер распределения радиоактивности в органах кроликов после введения меченного антигена Буавена или радиоактивной соли уксусно-кислого натрия, использованной при выращивании брюшнотифозных бактерий, был совершенно различен. Если бы изотопная группа отщепилась от антигена в организме, то различия в динамике распределения антигена и радиоактивной соли не удалось бы выявить.

Таким образом, все использованные контроли подтвердили, что на основании содержания радиоактивности можно судить о присутствии специфического О-антигена в органах иммунизированных животных.

Было вычислено, что по сравнению с подкожным введением в органы перорально иммунизированных животных — печень, селезёнку, лимфатические узлы — попадает в 100—500 раз меньше антигена при расчете на введённую дозу. Это может служить теоретическим объяснением, почему для достижения равного иммунологического эффекта для пероральной вакцинации требуются значительно увеличенные по сравнению с подкожными дозы вакцины. Вместе с тем этот тезис может быть обоснован только при установлении факта корреляции в антителопродуцирующих органах содержания антигена со степенью иммунного ответа.

В нашем исследовании при внутрикишечной и подкожной иммунизации кроликов радиоактивным антигеном Буавена одновременно с определением содержания антигена в различных органах — селезёнке, лимфатических узлах, ткани кишечника и аппендикса — определяли наличие антителообразующих клеток. Антителообразующие клетки выявляли по непрямому методу Jezne—Nordin (1963), используя баряни эритроциты, sensibilizированные О-антигеном, и заранее оттитрованный на отсутствие О-антител комплемент.

Была выявлена прямая зависимость между содержанием в органах, активно синтезирующих антитела, радиоактивного антигена и числом антителообразующих клеток. У подкожно иммунизированных кроликов в регионарных лимфатических узлах коэффициент корреляции между этими показателями составлял $+0,9$ на 3 день после иммунизации. В селезёнке этой группы кроликов он равнялся $+0,8$. Аналогичная зависимость была установлена для селезёнки и лимфатических узлов у внутрикишечно иммунизированных кроликов. Вместе с тем было показано, что внутрикишечная иммунизация, обеспечивающая большее и более раннее накопление антигена в ткани желудочно-кишечного тракта, сопровождается и более ранним накоплением антителообразующих клеток в лимфоидной ткани кишечника, аппендикса и в брыжеечных лимфатических узлах (Рис. 3).

Так, из результатов, приведённых на рисунке 3, следует, что в ткани тонкой кишки кроликов, иммунизированных за 24 часа до опыта, содержание антигена и антителообразующих клеток было выше после внутрикишечной иммунизации по сравнению с подкожной. В селезёнке и лимфатических узлах эти показатели были выше у подкожно иммунизированных кроликов.

Более раннее накопление антителообразующих клеток лимфоидными элементами желудочно-кишечного тракта у энтерально иммунизированных животных по сравнению с иммунизированными подкожно было установлено не только при введении меченого антигена Буавена, но также при использовании других тифо-паратифозных вакцин. Это было показано в наших

опытах при иммунизации внутрикишечно кроликов гретыми брюшнотифозной и паратифозной В моновакцинами, нерадиоактивным антигеном Буавена *S. typhi*. Примером могут служить данные, приведенные на рисунках 4 и 5, о динамике антителообразующих клеток в органах кроликов. Внутрикишечно кроликам вводили 40 млрд микробных клеток тифозной или паратифозной В вакцин, подкожно — 1 млрд микробных клеток.

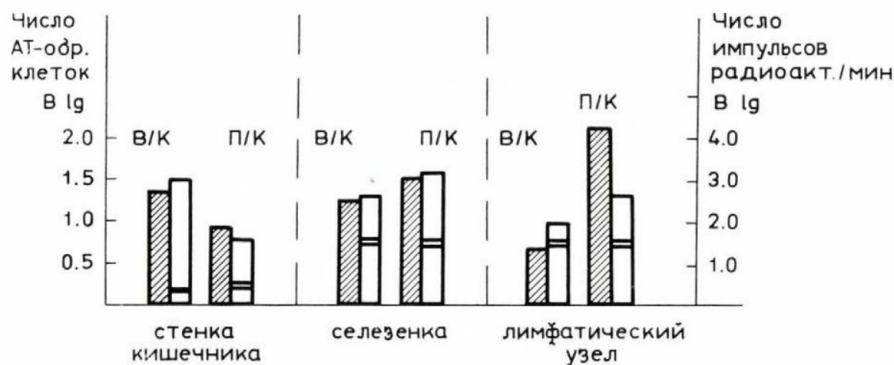


Рис. 3. Соотношение радиоактивности и числа антителообразующих клеток в органах кроликов через 24 часа после внутрикишечной или подкожной иммунизации радиоактивным антигеном Буавена *S. typhi*. ▨ радиоактивность, □ содержание антителообразующих клеток.

Внутрикишечная иммунизация обуславливала статистически достоверное увеличение числа антителообразующих клеток в стенке тонкой кишки, аппендикса и ткани брыжеечных узлов через 1—2 суток после введения вакцины и повышенное их содержание в этих органах до 14 дня наблюдения. После подкожной иммунизации кроликов антителообразующие клетки вначале накапливались в селезенке и регионарном (подколенном) лимфатическом узле, и только на 3—4 день число их значительно увеличилось в стенке кишки и аппендикса. Таким образом, энтеральная иммунизация обеспечивает более ранний синтез антител в области проникновения возбудителя кишечных инфекций. Развитие местных защитных реакций сопровождалось появлением генерализованного иммунного процесса у внутрикишечно иммунизированных кроликов — появлением антителообразующих клеток в селезенке, различных группах лимфатических узлов и нарастанием титра антител в сыворотке крови. (Рис. 5). Следовательно, внутрикишечное введение вакцин, так же как и подкожное, приводит к возникновению местных и общих иммунологических реакций. Однако местные реакции у энтерально вакцинированных животных появляются раньше, чем при

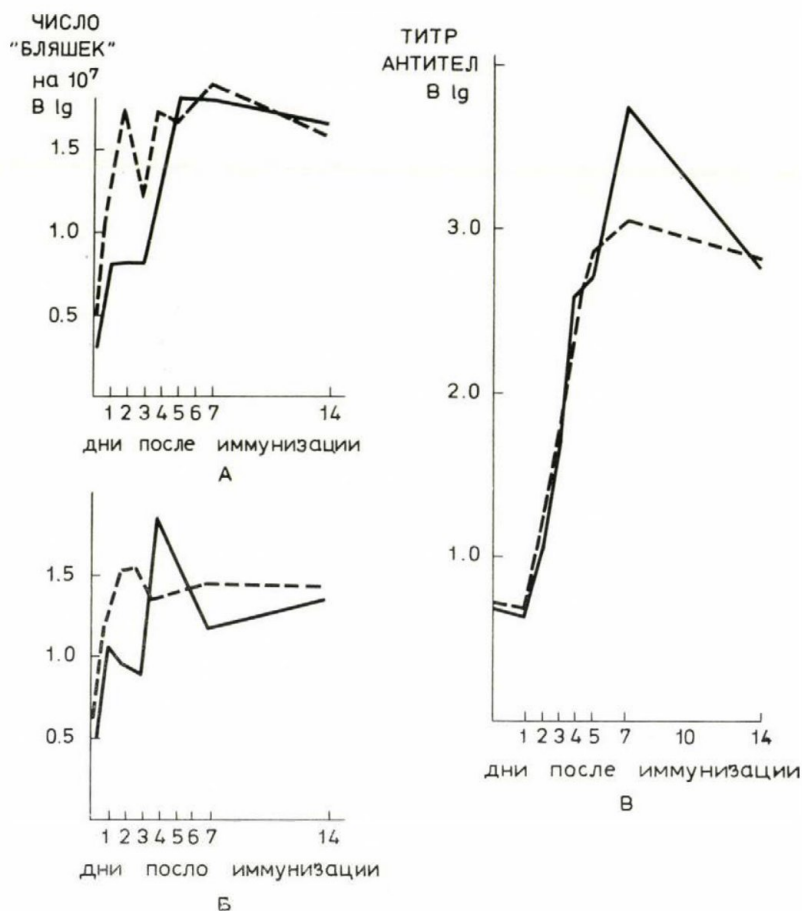


Рис. 4. Динамика антителиобразующих клеток и титра антител в органах кроликов, иммунизированных внутрибрюшинно или подкожно гретой брюшнотифозной вакциной: А — число антителиобразующих клеток в расчете на 10^7 лейкоцитов стенки тонкой кишки; Б — число антителиобразующих клеток в расчете на 10^7 лейкоцитов стенки аппендикса; В — титр антител в сыворотке крови кроликов, иммунизированных — — — внутрибрюшинно; ————— подкожно.

подкожном введении антигена. Это подтверждает необходимость энтерального введения вакцин для быстрого создания «иммунитета входных ворот».

Таким образом, результаты проведенных экспериментов показали, что энтеровакцинация против тифо-паратифозных инфекций наряду с уже известными достоинствами — простотой введения, безболезненностью, отсутствием тяжелых побочных реакций — обладает ещё одним существенным преимуществом, а именно: она обуславливает более быстрое развитие местных защитных реакций в лимфоидной ткани желудочно-кишечного тракта

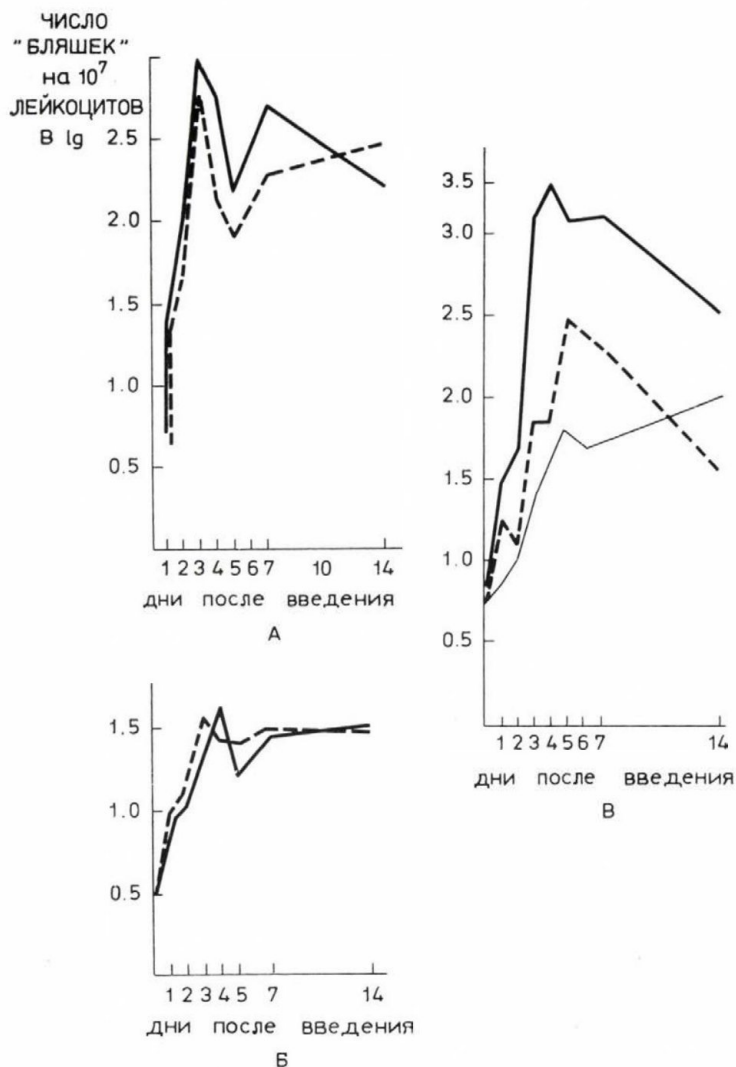


Рис. 5. Динамика антителообразующих клеток в лимфоидных органах кроликов, иммунизированных внутрикишечно или подкожно гретой брюшнотифозной вакциной (в расчете на 10^7 лейкоцитов органа). А — в селезенке; Б — в брыжеечных лимфатических узлах; — — — после внутрикишечной иммунизации; ————— после подкожной иммунизации; В — в подколennых лимфатических узлах; ————— в регионарном после подкожной иммунизации; ————— в отдаленном после подкожной иммунизации; — — — в подколленном после внутрикишечной иммунизации.

по сравнению с подкожной вакцинацией. Это имеет большое защитное значение, учитывая патогенез кишечных инфекций, при которых внедрение возбудителя происходит через слизистую оболочку желудочно-кишечного тракта.

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SAFETY TESTS IN ADULTS AND CHILDREN WITH LIVE ORAL TYPHOID VACCINE

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Summary. Studies on 1104 adults and 622 children have shown that streptomycin-dependent salmonellae given orally in doses of 60×10^9 per child, and 180×10^9 per adult were tolerated well in both groups. Additional studies are needed to confirm these findings prior to the use of such a strain as a vaccine in large-scale field trials.

Although parenterally administered vaccines have been shown to produce a fair degree of protection against typhoid fever, many studies in recent years have led to the conclusion that a better method of vaccination would be desirable. Oral vaccination was one logical alternative. The following advantages could be expected from orally applied vaccines:

- their simplicity of preparation and ease of administration;
- the absence of certain hazards associated with the administration of parenteral vaccines;

- and, finally, oral vaccines might offer the additional advantage of a direct stimulation of local intestinal immune mechanisms. The first line of defence against systemic invasion by typhoid bacilli should be the gastrointestinal tract. In typhoid carriers intestinal multiplication may occur without development of the disease, which suggests that local factors play an important role in conferring protection against manifest disease. It is supposed that a direct stimulation of intestinal immune mechanisms can be achieved either with oral killed or with oral live vaccines. Studies with both oral vaccines against typhoid as to reduction of the infection rate were encouraging but are still yielding conflicting results so that further extensive studies with both vaccines are needed. But let us call attention to the Report of a WHO Scientific Group on Oral Enteric Bacterial Vaccines: "Recent studies suggest that resistance to this group of systemic intracellular infection is better achieved with living than with killed vaccines. Living vaccines have the effect of restricting the spread of virulent organisms from the intestinal tissue . . ." [1].

On the other hand, preparation of killed vaccines requires exposure of the bacteria to physical or chemical agents which may destroy the antigenic characteristics necessary for stimulation of the protective antibody.

One of the major arguments in favour of killed oral vaccines is the safety of such vaccines.

IVERSON and WAKSMAN [2] were the first to call attention to the feasibility of using streptomycin-dependent micro-organisms in applied bacteriology. OLITZKI and SZENBERG [3] developed a streptomycin-dependent mutant of *Brucella abortus* that was employed in parenteral vaccination of animals and man. OLITZKI and OLITZKI [4] produced immunizing agents against cholera which gave good results when tested in laboratory rodents. MEL and associates [5-10] have developed streptomycin-dependent strains of *Shigella flexneri* 1, 2a, 3 and 4 as well as *Shigella sonnei* and used them as oral vaccines in extensive field trials. REITMAN [11] showed the innocuity of the streptomycin-dependent mutant of *Salmonella typhi* for mice and rabbits when administered parenterally, the antigenicity and immunogenicity of the dependent mutants administered as a live vaccine, and the stability by failure to obtain reversion *in vitro* and *in vivo*.

This report presents some data of the safety tests with a live oral streptomycin-dependent *S. typhi* vaccine in adults and children.

Our streptomycin-dependent Sm-D organism is a one-step mutant derived from strain Ty₂. For optimum growth the Sm-D strain requires 400 µg of dihydrostreptomycin sulphate per ml of nutrient medium and grows also on medium with 40 to 2000 µg. On media with 20 and with 2500 to 3500 µg per ml the mutant gives tiny colonies. Below 20 and above 3500 µg/ml of streptomycin no growth could be observed.

On media with 400 µg/ml of streptomycin, the colonies are normal in size and appearance. These Sm-D salmonellae were carefully tested and found to be indistinguishable from those of streptomycin-sensitive virulent parents even by Vi-antigen content. On media without streptomycin a trace of growth occurred on the point of heaviest inoculation. This is called residual growth but no colonies can be seen. Residual growth appears as long filaments. The Sm-D mutants in the absence of streptomycin are growing for a few generations in long filaments without cell division. They are inhibited in their ability to form septa. Streptomycin is required to form septation.

Like the sensitive parent strain they are very sensitive to kanamycin and chloramphenicol, relatively sensitive to ampicillin, terramycin and penicillin and resistant to erythromycin.

Administered to mice orally in doses of 10⁷ organisms, the mutant could be recovered from the stools for one day or two days, always on media with streptomycin. Injected intraperitoneally to laboratory animals in doses of 10⁷ to 10⁸ organisms, they disappeared from the abdominal cavity after a day or two but persisted in the liver and spleen for 20 or 21 days without any evidence of multiplication. From the liver and spleen of sacrificed mice only Sm-D organisms could be recovered and in no case was noted a reversion to streptomycin independence.

Studies in adults. The failure to recover, for the whole period of 20 days,

from the liver and spleen of the inoculated mice any but dependent salmonellae, seemed to justify an extension of experiments on volunteers. The first two volunteers were from our Institute. They ingested 20, 40 and 50×10^9 live Sm-D salmonellae in the first, second and third dose, respectively, at 5 day intervals. The number of individuals in the safety studies was increased to 20 volunteer students. Eight of them were hospitalized during the study at the Department for Infectious Diseases, Military Medical Academy, and received doses of 30, 40 and 50×10^9 organisms. The other 12 subjects were kept under daily control at the outpatient clinic and received 20, 50 and 60×10^9 live Sm-D salmonellae. All were examined daily during the vaccination period and 14, 20 and 30 days after the last dose of vaccine. All these volunteers were tested for shedding of the vaccinal strain in the faeces and for serum antibody tit. res. Daily faecal samples were plated onto SS agar, EMB agar and broth agar, all containing $400 \mu\text{g}$ per ml of streptomycin and on the same media without streptomycin, in order to detect reversions. The next was a group of 300 adults followed by a group of 782 adults. They received vaccines containing 50, 60 and 70×10^9 organisms.

All the subjects involved in these studies were 20–22-year-old healthy males. None of them had a history of typhoid fever or previous vaccination against typhoid fever.

In these studies lyophilized vaccine was used. After rehydration appropriate dilutions were made to contain the desired number of viable cells in 20 ml of the vaccine. The vaccine was administered with and without sodium bicarbonate pretreatment. In one group of adults under study streptomycin was added to the vaccine.

On the 1104 individuals involved in these studies, none developed any intestinal or systemic reaction. In no case was fever or diarrhoea noted.

Administration of the vaccine to the 20 volunteers induced no significant changes in body weight, temperature, blood pressure, white and red blood cell count, blood haemoglobin, erythrocyte sedimentation rate or differential white cell formula. No pathological changes were revealed by the results of urine analysis, electrophoretic serum protein pattern or functional liver tests.

From 300 faecal specimens taken from the 20 volunteers, and 840 from the adult subjects, in other words from a total of 1140 faecal specimens, only the vaccinal Sm-D salmonellae could be recovered and in no case was found a reversion to streptomycin sensitivity or to streptomycin resistance.

Like with Sm-D shigellae, sodium bicarbonate pretreatment enhanced the intestinal transit of Sm-D salmonellae. However, the mean duration of shedding, even after sodium bicarbonate pretreatment, amounted approximately to two days and was shorter than that observed with Sm-D shigellae. Addition of streptomycin extended the shedding period of the vaccinal Sm-D salmonellae.

In blood samples of the 20 vaccinated volunteers and of additional 30 adults, the humoral antibody response was as follows. Of the 50 subjects receiving 120 to 180×10^9 live organisms only 10 (20%) showed a fourfold rise in anti O agglutinins, 24 (48%) high titres against H antigens, and 32 (64%) a significant rise of Vi agglutinins.

Studies in children. The purpose of this part of the study was to find out, by evaluation of adverse reaction rates, how children were tolerating an oral live salmonella vaccine. Shedding of the vaccinal strain, the possible appearance of revertants in the faeces of vaccinees, and the extent of serological conversion were also studied.

School and preschool children of both sexes, aged from 5 to 13 years, were involved in this study. The distribution of vaccinated children by age and sex is shown in Table I.

Table I
Distribution by age and sex of children vaccinated with typhoid vaccine

Age, years	No. of children					
	vaccinated			placebo		
	♂	♀	total	♂	♀	total
5	25	20	45	18	19	37
6	33	40	73	35	42	77
7	36	36	72	34	41	75
8	33	37	70	36	36	72
9	39	29	68	41	26	67
10	42	44	86	41	46	87
11	23	33	56	20	34	54
12	46	42	88	44	40	84
13	30	34	64	33	36	69
Total	307	315	622	302	320	622

Of 1244 children, 622 received Sm-D vaccine, and 622 were controls randomly selected and treated with placebo. Vaccine was administered in 20 ml of milk, containing 1.5 g sodium bicarbonate and $19, 22$ and 25×10^9 live Sm-D salmonellae administered at three day intervals. Placebo consisted of 20 ml of milk with 1.5 g of sodium bicarbonate.

Following each dose the children were closely observed for vomiting, diarrhoea (defined as 2 or more loose stools in 24 hr), abdominal pain and fever. Daily observation of the children continued until 14 days after the last ingested dose. The children were seen by paediatricians 20, 30 and 60 days after the last ingested dose.

All loose stools were cultured. During the whole vaccination period, stools from 155 randomly selected children (25% of the vaccinated children) were cultured daily on two media, one with and the other without streptomycin. A total of 29 children were tested for agglutination titres against *S. typhi*.

Table II

Postvaccinal reactions following administration of three doses of typhoid vaccine

Dose		I	II	III	Total
Type of reaction					
Vomiting	vaccine	2	1	2	5
	placebo	2	—	2	4
Diarrhoea	vaccine	1	1	2	4
	placebo	2	4	2	8
Abdominal pain	vaccine	4	—	6	10
	placebo	2	—	3	5

No adverse effects were seen in any of the children. Not a single day of absenteeism caused by the vaccine or any significant postvaccinal reaction was noted. As it can be seen in Table II, postvaccinal reactions consisted in vomiting (0.8% in the vaccinated group against 0.6% in the control group), diarrhoea (0.6% against 1.3%), and abdominal pain (1.6% against 0.8% in the controls). All these symptoms were mild and disappeared spontaneously in a few hours.

No pathogens were found in the loose stools of children with postvaccinal reaction (diarrhoea). From a total of 1555 faecal specimens cultured from vaccinated children only streptomycin-dependent organisms could be recovered and no reversion to independence was found. The mean duration of shedding of the vaccine strain was approximately two days; on the first day after vaccination 79% (133/155) of the children, on the second day, 68.8% (106/154), on the third day, 18.7% (29/155) and on the fourth day 4.5% (7/155) of children shed the vaccinal strain.

Rates of seroconversion in children showed to be comparable to those encountered in adults.

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ИСПЫТАНИЕ ЭНТЕРАЛЬНЫХ ТИФО-ПАРАТИФОЗНЫХ ВАКЦИН В ЭКСПЕРИМЕНТЕ И НА ЛЮДЯХ

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MESHALOVA, A. N., KURLOVA, V. I., YANISKER, G. YA., LAVROVSKAYA, V. M., BLANT, M. E., NEGINA, YU., P.: **Animal and human studies on oral vaccination against typhoid and paratyphoid fever.** Oral typhoid vaccines prepared from extracts and heated bacteria showed a low reactivity and high immunogenicity. Field trial with combined typhoid-paratyphoid B (1 : 1) vaccine indicated that the immunogenicity of the typhoid component increased 3.6 times for the heated antigen and 6.3 times for the extract as compared to the effectiveness of the corresponding monovalent typhoid vaccines. The immunogenicity of the paratyphoid B component was the same in the monovalent as in the combined vaccine.

Резюме: В работе приведены результаты изучения увеличенных доз тифозных гретой и химической вакцин при пероральном применении на ограниченной группе детей от 3 до 15 лет и взрослых. Проведение строго контролируемого опыта показало, что энтеральные вакцины имеют слабую реактогенность и достаточно высокую иммунологическую эффективность. Иммунизация людей приводила к образованию гуморальных антител к О-, Ви- и Н-антигенам и обуславливала нарастание превентивной активности сывороток к О- и Ви-штаммам. Полученные данные являются основанием для рекомендации этих вакцин к испытанию в большом эпидемиологическом опыте в районах с повышенной заболеваемостью брюшным тифом. Кроме того приведены экспериментальные данные изучения иммуногенной активности ассоциированной тифо-паратифозной химической и гретой дивакцины.

Установлено, что ассоциация гретой тифозной вакцины с паратифозной В (в соотношении 1 : 1) повышает иммуногенность тифозного компонента в 3,6 раза в сравнении с моновакциной; химическая тифо-паратифозная вакцина (в соотношении 1 : 2) обуславливает повышение иммуногенности тифозного компонента в 6,3 раза в сравнении с тифозной моновакциной. Иммуногенность паратифозного компонента при ассоциации его с тифозным не изменялась.

Полученные данные свидетельствуют о возможности изготовления тифо-паратифозных дивакцин с применением уменьшенных доз тифозного компонента.

Проведенные ранее исследования, целью которых было выявить возможность приготовления иммуногенной тифозной вакцины для энтерального применения, показали, что гретая вакцина, приготовленная в виде драже, обладает достаточно высокими иммуногенными свойствами, вызывая образование антител и повышение превентивной активности сывороток у лабораторных животных (мыши, кролики, обезьяны).

При иммунизации людей эта вакцина не вызывала серологических сдвигов, титр антител и превентивные свойства сыворотки не повышались. В то же время испытание этой вакцины в большом эпидемиологическом опыте выявило статистически значимую разницу в заболеваемости среди

привитых и контрольных групп. В первые 3 месяца после прививок $P = 0,05$, в последующие 10 месяцев заболеваемость среди привитых была ниже, чем в контроле, но различия не имели статистической значимости [1—3]. Эти данные подтверждали эпидемиологическую эффективность энтеральной тифозной вакцины, но защитный эффект ее был не столь продолжителен. Возникла необходимость изучения энтеральных вакцин разных способов изготовления, в увеличенных дозах.

В настоящей работе приводятся данные изучения двух тифозных вакцин и двух тифо-паратифозных дивакцин. Гретая и химическая энтеральные моно-вакцины готовились в виде драже из одной микробной массы с использованием производственных штаммов Ту₂4446 брюшнотифозной культуры и № 42 паратифозной В культуры.

В опытах проверки иммуногенности вакцин были использованы мыши (беспородные) весом 12—14 г. Разведения вакцин проводили с интервалом кратности, равным 5—10. Мышей иммунизировали перорально и через 10 дней после иммунизации заражали путем внутрибрюшинного введения тифозной культуры того же штамма, который был использован для приготовления вакцины. Параллельно с титрованием эффективной дозы вакцин проводили титрование культуры. За состоянием мышей наблюдали в течение 3 суток, после чего определяли ЕД₅₀ вакцины и ЛД₅₀ культуры.

После приготовления и проверки в лаборатории каждая серия вакцины проходила контроль стерильности и иммуногенности в Государственном контрольном институте им. Тарасевича, после чего использовалась для изучения в ограниченном эпидемиологическом опыте. Предварительно вакцина была проверена на безвредность и реактогенность на добровольцах. В качестве плацебо было приготовлено сахарное драже, не содержащее вакцины. Вакцины и плацебо были закодированы.

Для проведения эпидемиологического опыта были взяты дети от 3 до 15 лет и взрослые, не получавшие ранее прививок против брюшного тифа. Все лица перед прививкой обследовались врачами с измерением температуры и в момент вакцинации были здоровы. Разбивку прививаемых контингентов на группы проводили случайно выборочным методом, который позволил получить равноценные группы прививаемых как по возрастному составу, так и по бытовым условиям, физическому развитию и перенесенным заболеваниям.

Реакции на прием вакцины учитывали через 6, 9, 24 и 48 часов после каждого приема вакцины. У привитых изучали температурную реакцию и клинические проявления в виде общего недомогания и явлений со стороны желудочно-кишечного тракта — боли в кишечнике, тошноты, рвоты, изменения характера стула. После первого и после второго приема вакцины проводили тщательный опрос прививаемых с целью выявления реакции. Наблюдения за привитыми проводили в течение двух недель.

У ограниченной группы привитых изучали образование антител и защитные свойства сывороток.

Наибольшее число реакций было выявлено после первого приема как химической, так и гретой вакцины, а также плацебо. Все реакции у привитых возникали в первые 6—9 часов после приема вакцины, они были слабые и кратковременные. По истечении 24—48 часов реакции наблюдались в единичных случаях. После второго приема вакцины реакции были отмечены в меньшем количестве, все они были слабые и кратковременные.

При проведении сравнительного анализа возникновения реакций на прививку вакцинами и плацебо у разных возрастных групп не удалось установить статистически значимых различий между реакциями на вакцину у детей разного возраста, а также между реакциями на вакцину и плацебо в каждой возрастной группе. В то же время у взрослых при наличии реакций на вакцину реакции на плацебо не выявлялись.

Таким образом, несмотря на применение больших доз вакцины, реакции у детей и взрослых были слабые и кратковременные. Все это свидетельствует о том, что химическая и гретая энтеральные вакцины, изготовленные в виде драже, слабо реактогенны. У 40 взрослых изучено 160 сывороток и у 60 детей — 120 сывороток, в которых определены серологические реакции и защитные свойства (Мешалова с соавт., 1972).

У всех взрослых, привитых химической и гретой вакцинами, наблюдалось статистически значимое повышение титров антител (2—4 раза) в отношении О-, Ви и Н-антигенов. В сыворотках детей выраженное нарастание титров антител (3—4 раза), имеющее достоверность, было отмечено лишь к О-антигену при иммунизации как гретой, так и химической вакциной, к Ви- и Н-антигенам антитела выявлялись в низких титрах. В контрольной группе, у привитых плацебо, повышение титров О, Ви- и Н-антител не отмечалось.

Результаты исследования защитных свойств сывороток привитых как химической, так и гретой вакциной, свидетельствуют о нарастании превентивной активности во все сроки исследования.

Результаты проведенного строго контролируемого опыта по оценке реактогенности и иммунологической эффективности химической и гретой энтеральных вакцин показали, что энтеральные вакцины имеют слабую реактогенность и достаточно высокую иммунологическую эффективность.

В то же время сравнительное изучение двух энтеральных тифозных вакцин — химической и гретой — не дает оснований для того, чтобы сделать заключение о большей иммунологической эффективности одной из них. Обе вакцины обладали достаточной иммуногенностью и обуславливали развитие иммунологической перестройки в организме привитых.

Эти исследования подтверждают полученные ранее данные о слабой реактогенности и выраженной иммунологической эффективности энтеральных вакцин при изучении их на добровольцах.

Это доказывает, что предложенные химическая и гретая энтеральные тифозные вакцины безвредны для человека.

Принятые для энтеральной иммунизации большие дозы вакцины вводились в один прием двукратно с интервалом 15—30 дней. Иммунизация людей приводила к образованию гуморальных антител к О-, Ви- и Н-антигенам и обуславливала нарастание превентивной активности сывороток к О и Ви-штаммам, что свидетельствует о возможности использования этих доз и схемы приема вакцины для проведения энтеральной вакцинации.

Сравнивая полученные результаты с данными предыдущего опыта, можно отметить, что увеличение разовой дозы вакцины и изменение схемы иммунизации обусловили развитие у привитых иммунологической реактивности. Надо полагать, что наличие у привитых подъема специфических антител и защитных свойств сыворотки является положительным фактором, дающим основание надеяться на получение длительной эпидемиологической защиты при массовой иммунизации, учитывая, что в предыдущем эпидемиологическом опыте применение небольших доз вакцины, не вызывающих серологических сдвигов, обусловило статистически значимую эпидемиологическую эффективность на протяжении трех месяцев.

Приготовленные энтеральные вакцины в виде драже с применением в качестве защитного вещества — пектина не требуют при приеме особой подготовки прививаемых. Техника проведения прививок проста, легко осуществляется и позволяет за короткий срок проводить прививки больших групп населения. Проведение прививок занимает мало времени, не требует специально обученного персонала, полностью исключает применение шприцев и игл, необходимых для осуществления подкожной иммунизации, или специальных распыливающих устройств и дозиметров, применяемых при аэрозольной вакцинации.

Простота и легкость проведения прививок энтеральными вакцинами может быть удобной в практике противоэпидемиологической службы, особенно при иммунизации детей раннего возраста и населения, находящегося в условиях чрезвычайного положения (наводнение, землетрясение, лучевое облучение и т. д.).

Таким образом, результаты проведенного опыта показали, что обе энтеральные вакцины, независимо от метода изготовления, химическая и гретая, безвредны, обладают слабой реактогенностью и достаточно высокой иммунологической эффективностью.

Полученные данные являются основанием для того, чтобы рекомендовать испытание этих вакцин в большом эпидемиологическом опыте в районах с повышенной заболеваемостью брюшным тифом, с целью выявления их эпидемиологической эффективности. Кроме того, иммунологическая эффективность энтеральных тифозных вакцин, простота и легкость осуществления прививок дают основания использовать энтеральный метод для экспресс

иммунизации в чрезвычайных условиях, связанных со стихийными бедствиями (землетрясение, наводнение и т. д.), когда населению грозит большая опасность заражения.

В экспериментальных условиях было установлено, что ассоциация брюшнотифозного и паратифозного В компонентов при изготовлении дивакцины повышают их иммуногенную активность.

Гретья тифо-паратифозная дивакцина, в которую оба компонента вводили в равных количествах (соотношение 1 : 1), обусловила повышение иммуногенности тифозного компонента. Средняя эффективная доза тифозного компонента дивакцины составила 0,8, а моновакцины — 2,9 млрд. микробных клеток, т. е. величина ED_{50} тифозного компонента дивакцины в 3,6 раза меньше ED_{50} моновакцины. Эта разница в дозах статистически значима. ED_{50} паратифозного компонента дивакцины была равна 1,1, а моновакцины — 1,5 млрд. микробных клеток; эти величины не отличались между собой.

Испытание иммуногенных свойств химической тифозной дивакцины было проведено при соотношении тифозного и паратифозного компонентов 1 : 2. Сравнение иммуногенности тифозного компонента дивакцины с тифозной моновакциной показало, что ED_{50} тифозного компонента дивакцины была в 6,3 раза меньше ED_{50} тифозной моновакцины при $P \leq 0,001$. Разница ED_{50} паратифозного В компонента дивакцины и моновакцины не имела статистической значимости. Увеличение дозы паратифозного В компонента в химической дивакцине в 5 раз по отношению к тифозному выявило повышение его иммуногенности в сравнении с моновакциной в 3,3 раза.

Изготовленные гретья и химическая дивакцины, в которых дозы компонентов были уменьшены: тифозного в 3—6 раз соответственно и паратифозного в 2 раза в сравнении с моновакцинами, при испытании иммуногенной активности показали высокую защитную способность.

Эти данные свидетельствуют о возможности изготовления энтеральных тифо-паратифозных вакцин с применением уменьшенных доз тифозного компонента.

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ИССЛЕДОВАНИЕ ИММУНОЛОГИЧЕСКИХ РЕАКЦИЙ МЕТОДОМ КУНСА В ОТВЕТ НА ЭНТЕРАЛЬНУЮ ВАКЦИНАЦИЮ ПРОТИВ БРЮШНОТИФОЗНОЙ ИНФЕКЦИИ

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PERSHIN, B. B., LEBEDEV, K. A., NOVIKOVA, T. A.: **Application of the Coons test in studies on immune reaction to oral typhoid vaccination.** Rabbits and guinea pigs were vaccinated orally, intra-intestinally and parenterally with living *Salmonella typhi* bacteria (strain 5501), with their extract and with living Vi-antigen-containing *Citrobacter* culture (strain 5396/38). Antibody-producing cells were present in the greatest number in the spleen and in remote lymph nodes. In the lymphoid tissue of the intestinal tract a non-specific reaction manifesting in the accumulation of large amounts of globulin-producing cells and a definite activity of the secondary follicles were observed. The immune reaction in the gastrointestinal tract showed no parallelism with the antibody titre of the serum. The results indicated that there was a basic difference between immune reaction developing after enteral vaccination and immunity produced with parenteral injection.

Резюме: В исследованиях непрямым методом Кунса на кроликах и морских свинках, иммунизированных энтерально, внутрикишечно, энтерально-подкожно и парентерально, против брюшнотифозной инфекции живой культурой брюшнотифозных бактерий (штамм 5501), антигеном из этой культуры, живой культурой цитробактер (5396/38), содержащей Ви-антиген, и дифтерийным анатоксином, показано, что наибольшие количества антителопродуцирующих клеток были отмечены в отдаленных лимфоузлах и в селезенке. В лимфоидной ткани кишечника преобладала неспецифическая реакция в виде значительных количеств глобулинпродуцирующих клеток и сильной активации центров размножения вторичных фолликулов. Иммунологическая реакция в желудочно-кишечном тракте практически не зависела от уровня титров сывороточных антител.

Результаты проведенных исследований позволяют прийти к выводу о значительных отличиях иммунологической реакции, развивающейся в результате энтеральной иммунизации в сравнении с парентеральной.

Анализ контролируемых испытаний, проведенных под руководством ВОЗ, показал, что ни один из лабораторных тестов оценки вакцинных препаратов против кишечных инфекций не отвечал эпидемиологической эффективности [1].

Этот факт позволил вновь обратиться к теории местного иммунитета, выдвинутой Безредка, и на новых методических основах заняться изучением иммунитета, формирующегося в лимфоидной ткани желудочно-кишечного тракта.

В настоящее время благодаря исследованиям [2—7] показано, что ведущую роль в специфической резистентности играют секреторные иммуноглобулины. Однако в вопросе изучения специфичности иммунного ответа на антигенную стимуляцию существует много спорных и нерешенных вопросов. Так, например, до настоящего времени не находит объяснения феномен ин-

дукции антигеном синтеза специфических антител и неспецифических иммуноглобулинов, которые при полной структурной идентичности различаются в способности взаимодействовать с антигенной субстанцией, использованной для иммунизации [8].

По данным Краснопрошиной с соавторами [9], использовавших метод Ерне для исследования антителопродуцирующих клеток у кроликов в процессе энтеральной иммунизации, происходит ярко выраженное нарастание иммунокомпетентных клеток, дающих зону гемолиза. В других исследованиях [10] показано, что использование метода локального гемолиза не позволяет с уверенностью говорить о том, что в этом случае исследователь регистрирует только антителопродуцирующие клетки. Так, при первичном иммунологическом ответе выявляются клетки, не имеющие отношения к антителообразованию в 20 процентах случаев, а при вторичном в 40 [10].

В наших исследованиях мы попытались применить более чувствительный метод Кунса для изучения лимфоидной ткани экспериментальных животных (кроликов и морских свинок) в процессе вакцинации против брюшнотифозной инфекции.

В качестве вакцин использовали: живую культуру брюшнотифозных бактерий (штамм 5501), антиген из этой же культуры, полученный по методу Вестфала, живую культуру цитробактер (штамм 5396/38), содержащую Ви-антиген, и дифтерийный анатоксин.

В разных сериях экспериментов контролем служили интактные животные. Внутрикшечную иммунизацию проводили однократно и двукратно, энтеральную — однократно, двукратно, трехкратно, шестикратно и пятнадцатикратно. Парэнтеральную иммунизацию осуществляли общепринятыми методами. В исследованиях были использованы разные дозировки антигенов и живых культур. В процессе вакцинации животных забивали на второй, четвертый, седьмой, одиннадцатый и двадцать первый дни с момента начала иммунизации. С периодичностью 2—5 дней у животных брали кровь для титрования сывороточных антител. В день окончания опыта животных обескровливали и исследовали: мезентериальные, подколенные лимфоузлы, стенку толстого и тонкого кишечника, селезенку и аппендикс. Из взятых образцов готовили парафиновые срезы по методу Санта-Мария для последующей люминесцентно-сéroлогической окраски [11].

В ответ на парэнтеральную и внутрикшечную иммунизацию животные отвечали достаточно высокими титрами сывороточных антител. Энтеральная иммунизация, в сравнении с подкожной, вызывала образование антител в меньших титрах. Причем, интенсивность подъема титров антител зависила от дозы и кратности иммунизации. Имунные сыворотки, полученные в результате энтеральной и парэнтеральной иммунизации, обладали выраженными защитными свойствами в опытах на мышах в сравнении с исходными и нормальными сыворотками.

При титровании гомогенизатов лимфоузлов, стенки кишечника, селезенки, аппендикса и копрофильтратов независимо от способа иммунизации выявлены сравнительно небольшие титры специфических антител.

Таким образом, энтеральная иммунизация в сыворотке крови, копрофильтратах и гомогенизатах органов вызывала подъем титров антител, которые были ниже титров в сыворотке крови после парентеральной вакцинации.

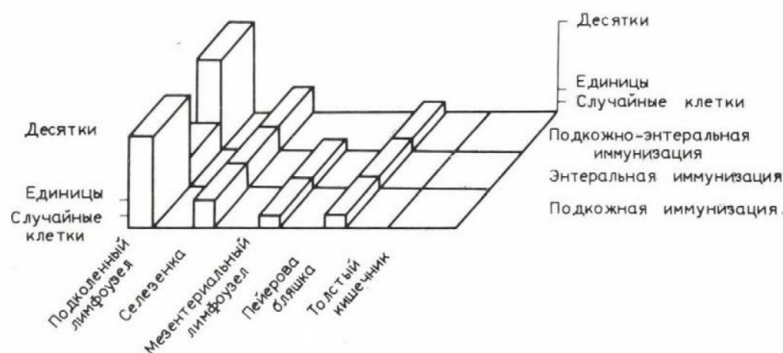


Рис. 1. Количество клеток, содержащих О-брюшнотифозные антитела, приходящихся на срез лимфоидных органов кролика при разных методах иммунизации

Исследование специфической иммунологической реакции методом Кунса показало, что при разных методах иммунизации, испытанными в наших опытах антигенами, наибольшее количество антителопродуцирующих клеток мы смогли обнаружить в лимфоузлах и селезенке. В мезентериальных лимфоузлах, Пейеровых бляшках и в стенке тонкого и толстого кишечника были выявлены лишь незначительные количества антителопродуцирующих клеток, независимо от способа иммунизации. (Рис. 1).

При исследовании реакции глобулинпродуцирующих клеток лимфоидной ткани желудочно-кишечного тракта животных (стенка тонкого и толстого кишечника, Пейеровы бляшки, солитарные фолликулы) наибольшее количество глобулинпродуцирующих клеток было отмечено лишь при энтеральной иммунизации. (Рис. 2, рис. 3а и б.)

Подавляющее большинство лимфоидных клеток субэпителиального пространства тонкого кишечника у животных, иммунизированных этим способом, обнаруживали интенсивное свечение при окраске на глобулин.

Кроме того, энтеральная иммунизация подопытных животных, в отличие от других методов, вызывала более сильную активацию центров размножения вторичных фолликулов стенки тонкой кишки и мезентериальных лимфоузлов (Рис. 4, рис. 5а и б.)

Аналогичные исследования подколенных лимфоузлов показали низкий процент активных центров размножения вторичных фолликулов при энтеральной иммунизации. Сильная активация была выявлена лишь при подкожной иммунизации в лимфоузле, который являлся регионарным к месту введения антигена.

Вакцинация разными методами культурой цитробактер и дифтерийным анатоксином вызывала иммунологическую реакцию, аналогичную опи-

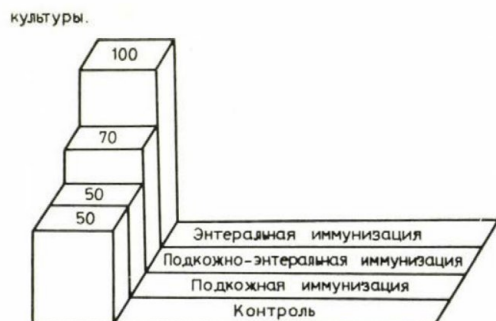
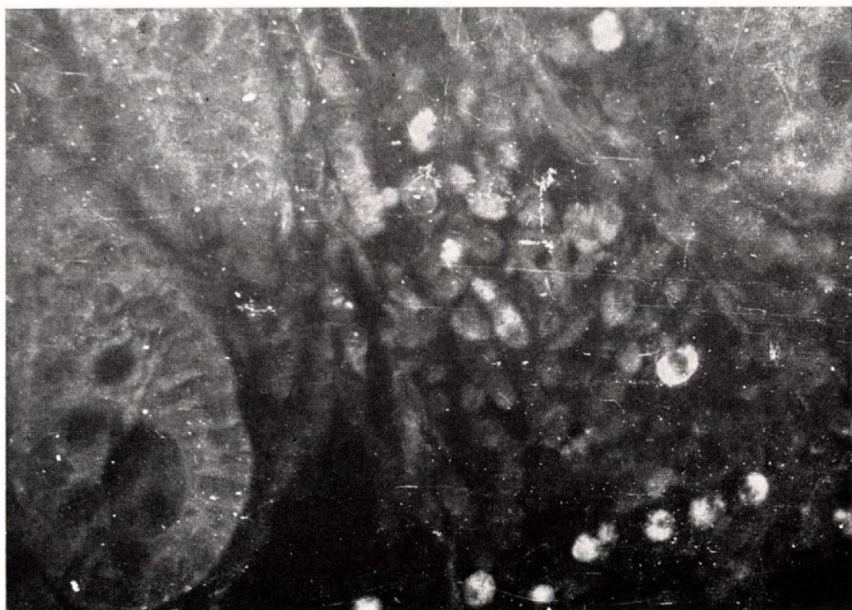


Рис. 2. Количество глобулинпродуцирующих клеток, приходящихся на одинаковую площадь собственного слоя слизистой тонкого кишечника кроликов, в ответ на энтеральную, подкожную и энтерально-подкожную иммунизацию живой брюшнотифозной культурой (штамм 5501) и О-антигеном из этой культуры

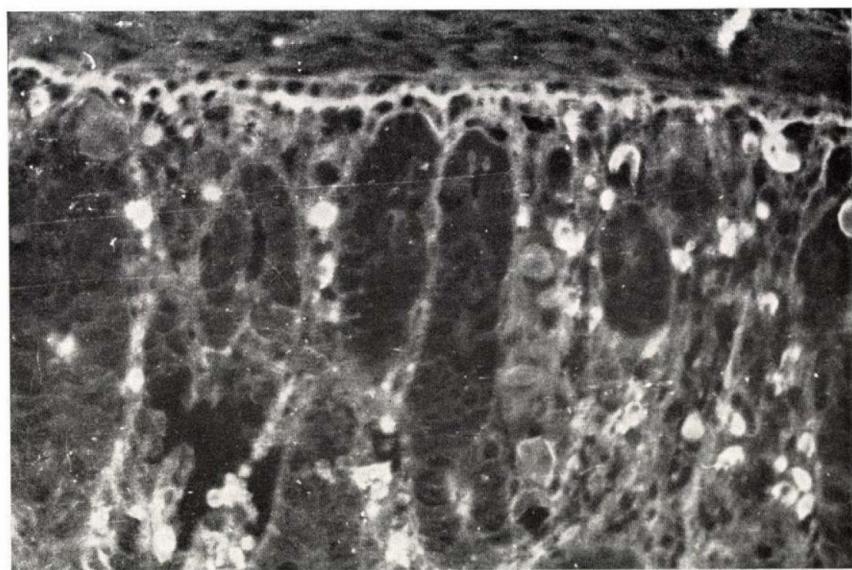
санной в отношении живой культуры брюшнотифозных бактерий и антигенного препарата из этого штамма.

Материалы настоящего исследования позволяют прийти к выводу о значительных отличиях иммунологической реакции, развивающейся при энтеральной вакцинации, в сравнении с парэнтеральной. Прежде всего это касается стимуляции поствакцинального иммуногенеза в лимфоидной ткани желудочно-кишечного тракта, которая составляет 70% всей лимфоидной ткани организма. Преобладание специфического компонента реакции в лимфоузлах можно объяснить соотношением антителопродуцирующих клеток к количеству лимфоидной ткани в лимфоузлах и в желудочно-кишечном тракте. Однако, результаты нашего исследования позволяют с известной степенью достоверности говорить о преобладании неспецифического компонента иммунного ответа над специфическим. Хотя в настоящее время нет сведений, позволяющих судить о том количественном уровне специфического иммунного ответа, необходимого для создания невосприимчивости к инфекции.

При обсуждении данного вопроса можно остановиться на работах [12, 13] по изучению трансплантационного иммунитета, где показано, что



(a)



(b)

Рис. 3. Глобулинпродуцирующие клетки в собственном слое слизистой тонкой кишки кролика в норме (а) и после энтеральной иммунизации (б). (Окраска на глобулин по Кунсу)

только 3—6% лимфоцитов, несущих специфические антитела, участвуют в отторжении трансплантата. Остальная часть клеток, принимающая участие в этом процессе, не содержит в своей структуре специфических антител к чужеродной ткани. Очевидно как в инфекционном иммунитете, так и в иммуногенезе, развивающемся к трансплантату, необходимо присутствие сравнительно небольшого количества специфических антителопродуцирующих клеток, участвующих в распознавании своего и чужого. Следующим этапом,

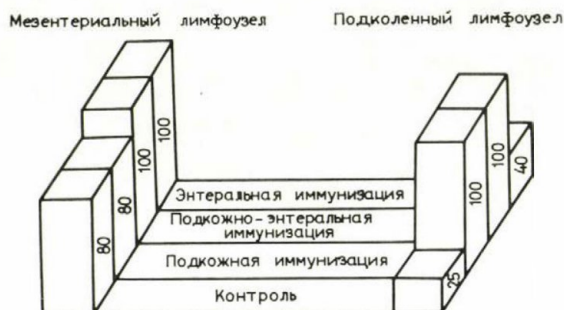
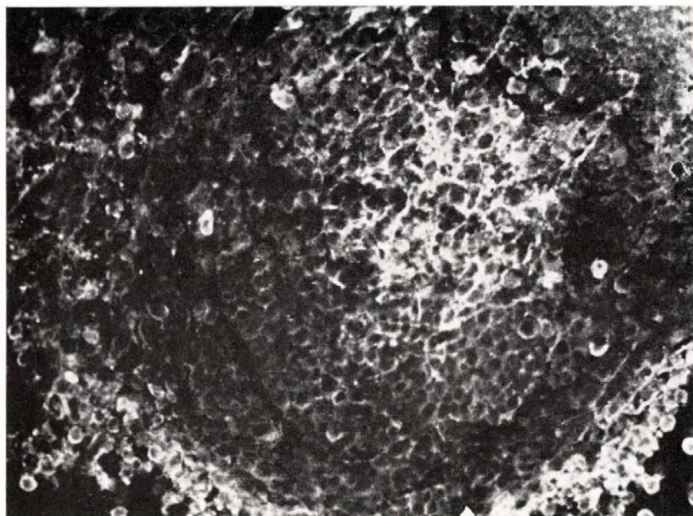


Рис. 4. Процент активных центров размножения вторичных фолликулов в подколенных и мезентериальных лимфоузлах кроликов в ответ на энтеральную, подкожную и энтерально-подкожную иммунизацию живой брюшнотифозной культурой (штамм 5501) и О-антигеном из этой же культуры

повидимому, включаются другие механизмы защиты, носителем которой могут быть лимфоидные клетки разного уровня специфичности.

Результаты исследований иммунитета, формулирующегося после энтеральной вакцинации, по методам Ерне и Кунса, повидимому, не находятся в принципиальном противоречии. В том и другом случае показан определенный уровень антителопродуцирующих клеток после введения антигена. Однако, методом Кунса выявлена значительная продукция неспецифических иммуноглобулинов. Можно предположить, что эти иммуноглобулины или их часть обладает овидностью, не улавливаемой методом Кунса. Кроме того, нельзя не учитывать биологического значения продукции иммуноглобулинов в кишечнике, несущих на себе антитела к другим антигенам разной природы, т. к. лимфоидная ткань желудочно-кишечного тракта имеет постоянный контакт с нормальной микрофлорой, с разными штаммами микроорганизмов, постоянно поступающих с пищей и разного рода пищевыми антигенами.

Настоящее исследование, по нашему мнению, находится в соответствии с результатами испытаний на добровольцах энтеральных вакцин против брюшного тифа и паратифов. В контролируемом опыте показано, что



(a)



(b)

Рис. 5. Активный (a) и неактивный (б) центры размножения в мезентериальном лимфоузле кролика. (Окраска на глобулин по Кунсу)

энтеральная иммунизация вызывает статистически значимое нарастание сывороточных иммуноглобулинов А и М в сравнении с подкожной иммунизацией [14]. Объяснением этому факту может служить лишь то обстоятельство, что при энтеральной иммунизации, в отличие от других методов, вовлекаются в процесс иммунологической реакции большие количества лимфоидной ткани.

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IMMUNIZATION OF INFANTS AGAINST ESCHERICHIA COLI ENTERITIS

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Summary. The immunogenicity and reactivity of a vaccine in tablets containing 0.5 mg antigen from each of the *Escherichia coli* strains O111:K58(B4), O55:K59(B5) and O86:K61(B7) has been studied in 62, one to eight months old, mainly dystrophic infants. Eight tablets (12 mg) daily proved to be completely harmless. Antigenicity of the vaccine was studied by testing serum and copro-antibodies. Serum antibodies were tested in ten days old chick embryos, while copro-antibodies were measured by passive immunization of mice. Suboptimum doses ($2-3 \times 3$ tablets) of antigen caused a fivefold rise in the protective titre of serum antibodies and this could further be increased by giving one tablet weekly. The immune response was polyvalent. Higher dose of antigen ($2-3 \times 8$ or 8×3) resulted in an 18-55-fold rise in titre, which was significant statistically. This antibody level could not be increased significantly by weekly revaccination. Vaccination provoked the appearance of copro-antibodies the protective effect of which was comparable to the serum antibody titre. Development of serum and copro-antibodies showed that revaccination was necessary for maintaining immunity. Preliminary experiments on mice showed that the immunogenic activity possessed by Boivin antigen was identical to that of the vaccine containing either killed or live germs. The experiments in children were carried out mainly with Boivin antigen, since its excellent efficacy in humans has been demonstrated in previous studies. It has also been demonstrated that as regards immunogenicity the killed corpuscular vaccine and the Boivin antigen was equivalent. In the course of an outbreak of *E. coli* enteritis in an infant ward Boivin vaccination afforded 60% protection. This will be discussed in a future paper.

The generally accepted view that for preventing *Escherichia coli* enteritis, oral immunization is only suitable, is supported by numerous data in the literature and our results obtained in mice.

In our investigations a suckling mice-pathogenic *E. coli* strain (O101:K30) behaved like strains of human *E. coli* dyspepsiae, as regards age, localization and the course of infection, and also as regards immunity. It was shown for example that protection was the most effective with oral passive immunization, when there is a concentration of antibodies in the intestine. This points to the obvious analogy with copro-antibodies, to be discussed later.

It is a matter of arguments whether killed or live vaccines should be used. Laboratory results obtained in serum samples of humans treated with Boivin type shigella vaccine were favourable. For further clarification we carried out experiments on mice, using killed and live suspensions and cell-equivalent Boivin antigen of the same strain. Immunity was tested by intraperitoneal infection with the mucine technique. According to the mean values obtained in several experiments, $1.3-1.7 \times 10^{10}$ cells represented the ED₅₀,

therefore the immunizing dose being the same, there was no significant difference between killed and live vaccine.

Table I

LD₅₀ of live and killed O111:K58(B4) antigens in mouse-test against 100 LD₅₀ of homologous strain

Boivin extract		Vaccine, germ count	
mg	germ equivalent	live	killed
0.278	1.4×10^{10}	1.3×10^{10}	1.7×10^{10}

Of the three vaccines the Boivin antigen was chosen, as its antigenicity proved to be potent in human trials with shigella antigen. The results obtained with this preparation will be discussed below.

Most authors studied the immunogenic effect of killed *E. coli* vaccines or antigens (LIETZ and RAETTIG [1]; OCKLITZ, MOCHMANN and co-workers [2-9]). There are few data on studies with streptomycin-dependent live strain (LINDE *et al.* [10-12]).

Boivin extracts prepared from the three most frequent *E. coli* dyspepsiae serogroups in Hungary, namely O111:K58(B4), O55:K59(B5) and O86:K61(B7) were used. The effectiveness of Boivin antigens was estimated by the haemagglutination inhibition test [13-15]. The antigens were given in the form of tablets each containing 0.5 mg antigen from each of the strains. Mice were fed with the antigens orally, by using a metal bougie, while infants received the antigen in tea before the morning meal, either at 5 day intervals or daily. After completing the course, 0.5 mg doses of antigen were given weekly for maintaining immunity. For immunological investigation, one blood sample was taken before and several samples after the immunization. To determine the titre of protective antibodies, serum samples were inoculated into 10-day-old chick embryos [16] *via* the vein of the chorioallantoic membrane. Then they were infected with the appropriate LD₅₀ of *E. coli* (50-75 LD₅₀; 50-100 germs). Copro-antibodies were studied in mice by using the intraperitoneal mucine technique.

Experiments in mice. Investigations were started in mice. The basic experiments revealed that Boivin antigen administered orally afforded protection against the infection. Protection ceased within a month, but small antigen doses given at 5 day intervals were sufficient not only to maintain but even to increase immunity. Protective copro-antibodies too were provoked and their effectiveness could also be increased by small antigen doses.

Observations in infants. First of all, the innocuity of the antigens was studied. It was concluded that of the vaccine doses several times higher than

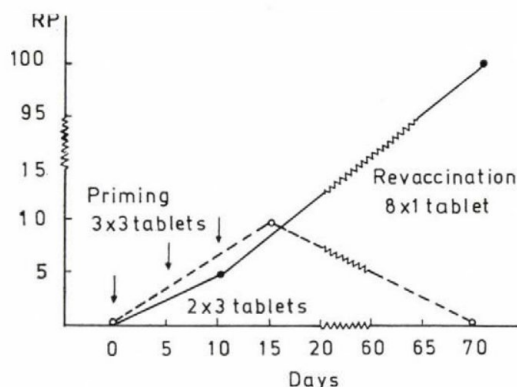


Fig. 1. Immunogenic effect of small doses of Boivin antigen and the duration of immunity with and without revaccination. Passive chick embryo test, *E. coli* O111:K58 (B4). Infectious dose, 25 LD₅₀; — revaccinated; - - - - not revaccinated

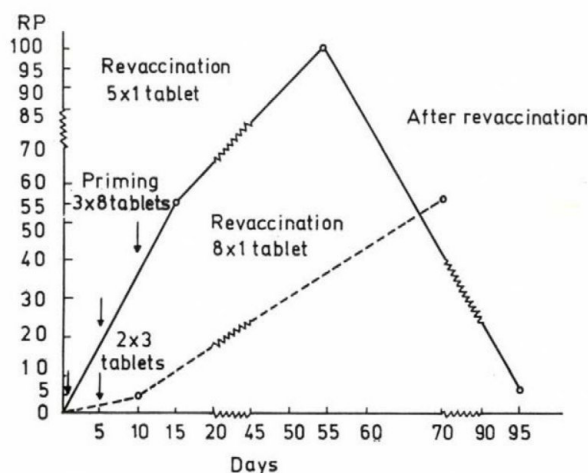


Fig. 2. Immunogenic effect of small and optimum doses of Boivin antigen given at 5 day intervals, and the duration of immunity. Passive chick embryo test, *E. coli* O111:K58 (B4). Infectious dose, 75 LD₅₀; — optimum dose; - - - - small dose

those used for immunization did not cause any side effect even in atrophic infants. Then the infants were immunized.

The protective antibody level increased 5–10-fold on giving small doses (2×3 or 3×3 tablets) of vaccine. Surprisingly, one tablet per week for eight weeks provoked a further 20-fold rise in protective titre. Without revaccination the titre fell rapidly (Fig. 1). The efficacy of revaccination was explained by the low dose of antigen used for basic immunization. Therefore, the effect of higher antigen doses (3×8 tablets) was studied.

Eight tablets given 3 times increased the titre 55-fold. By 5 revaccinations the protective titre was elevated to about twice this value, but according to

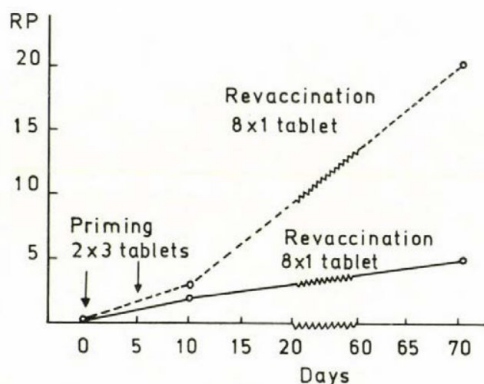


Fig. 3. Protective value of serum and copro-antibodies. Passive mouse protection test, *E. coli* O111:K58(B4). Infectious dose, 25 LD₅₀; — pooled faecal extracts; - - - - pooled sera

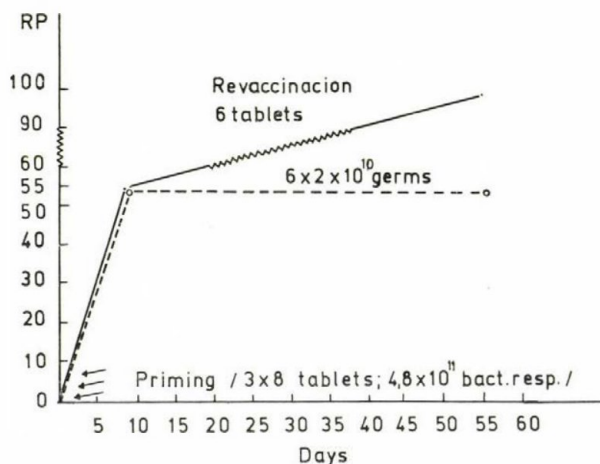


Fig. 4. Immunogenic effect of Boivin antigen and germ equivalent of corpuscular vaccine given on 3 consecutive days. Infectious dose: O111:K(B4) 75 LD₅₀; — Boivin; - - - - vaccine

statistical analysis it was of the same order of magnitude as after the basic immunization. This means that the increased doses of antigen applied for basic immunization ensured a level of protection which could not be increased significantly. Nevertheless, revaccination had to be continued, otherwise the protective titre decreased rapidly.

Determination of copro-antibodies was done in mice because of the toxicity of the faecal extracts for chick embryos. It was found that in vaccinated infants copro-antibodies had appeared after a small basic immunizing dose of antigen and on revaccination their titre increased significantly. The copro-antibody titre rose parallel with the serum antibody titre. The low values were

ascribed to the use of mice for this test. As the presence of copro-antibodies is known to be indicative of immunity in cases of dysentery and cholera, so their presence after immunization against *E. coli* enteritis had to be considered beneficial.

The special epidemiological nature of *E. coli* enteritis requires massive protection in the earliest period of life. In the present study the optimum dose of antigen (24 tablets; 12 mg antigen per type) given for three consecutive days resulted in the same level of immunity as that ensured by more prolonged immunization (Fig. 4).

As to formaldehyde killed vaccine, in adequate doses (4×10^{11} germs given either 6 or 3 times orally), it offered the same results as did the Boivin extract (Fig. 4).

The above immunizations were carried out in a children's hospital. The epidemiological data have confirmed our expectations, although we did not apply the optimum immunizing dose. Results achieved with such a dose will be presented in a future paper.

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EPIDEMIOLOGICAL-STATISTICAL EVALUATION OF ORAL VACCINATION AGAINST INFANTILE ESCHERICHIA COLI ENTERITIS

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Summary. In 1969—1971, in a children's hospital 627 infants were administered oral vaccine against *Escherichia coli* enteritis and 1040 infants received placebo. The vaccine was made of the Boivin extracts of freeze-dried *E. coli* O111, O55 and O86 as developed by RAUSS. One tablet contained 0.5 mg of each antigen. Vaccination included basic immunization with 3 tablets daily for 6 days (9 mg antigen) and booster immunization with 1 tablet weekly. In the period of observation 28 infants (4.5%) of the immunized group fell ill due to iatrogenic *E. coli* infection; this rate in the control group amounted to 79 infants (7.6%). The incidence of disease and the protective effect of vaccination depended on the age of the infants. Among the 0-month old, 6.6% of the immunized infants and 9.5% of the controls contracted the disease; this meant a protective efficacy of 30.5%. Of the patients between 1 and 2 months, 4.2% of the vaccinated and 9.3% of the non vaccinated fell ill; protective efficacy amounted to 54.8%. Among patients older than 2 months the incidence was 1.3% in the vaccinated group and 5.0% in the control group; protective efficacy was 74.0%. The less satisfactory results below 1 month of age were ascribed to the late cases of infection in the immunized group. Immunity lasted for one month. This is why basic immunization must be repeated at 2-week intervals and the dosage should be increased. Beyond 1 month of age the protective efficacy of vaccination was good, the risk of contracting the disease could be reduced to about one-third.

In 1969–71, controlled clinical trials were made with an oral vaccine at the Paediatric Department of the University Medical School, Szeged.

The vaccine made of Boivin extracts of freeze-dried *E. coli* O111, O55 and O86 was developed and kindly provided for the trial by Professor K. RAUSS, Institute of Microbiology, University Medical School, Pécs. One tablet contained 0.5 mg of each antigen. The tablets were given dissolved in tea. No side effect was observed after immunization [1–3].

Infants 0 to 12 months old, admitted to the hospital were randomly administered either vaccine or placebo. For basic immunization, 3 tablets were given daily for 6 days, thus a total of 9 mg antigen of each *E. coli* strain. Children staying at the hospital for a longer period received as a booster dose one tablet weekly. Patients completing the basic immunization schedule and receiving the booster dose within 14 days were regarded as immunized.

Questionnaires as to the history of the patient were filled in. Of the approximately 2000 questionnaires 1667 were evaluated statistically [4, 5]. Immunization of 627 children complied with the above principles, 1040 children served as controls.

E. coli hospital infections. On the 1667 infants, 107 (6.4%) fell ill after iatrogenic infection.

The incidence varied with the age of the patient and the duration of stay in hospital. It was 8.3% of the 0-month age group, 7.4% in the 1-2 months group, and 3.6% in those above 2 months of age.

With a hospital stay shorter than 30 days, 2.5% contracted *E. coli* enteritis, with a 1-2-month hospital stay the rate increased to 14.2%, and with more than 2 months in hospital, to 23.0% (Fig. 1).

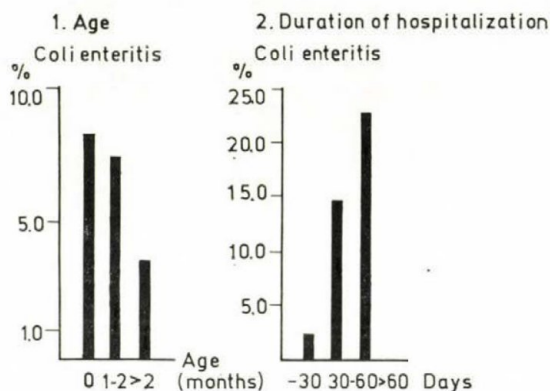


Fig. 1. Incidence of *E. coli* enteritis (per cent)

Thus, hospital infection threatened mainly patients of less than 1 month of age and subjected to long treatment. Thus, it is mainly these two groups which need to be immunized.

Epidemiological evaluation of the protective effect of immunization. The patients were classified into 4 groups: 1. Premature infants under 2500 g birth weight aged 0 month; 2. Mature newborns above 2500 g birth weight; 3. Infants 1 to 2 months old; 4. Infants above 2 months of age. Half of the patients belonged into groups 1 and 2, the other half was older.

Protective effect of the vaccine was calculated and based on the difference of disease incidence between the immunized and non-immunized groups.

(a) Of the 0-month-old immunized infants, 6.6% contracted the infection, versus 9.5% in the control group. Protective efficacy amounted to 30.5%.

(b) Among the 1-2-month old patients, 4.2% of the vaccinated and 9.3% on the non-vaccinated fell ill. Protective efficacy was 54.8%.

(c) Of the patients older than 2 months, 1.3% of the vaccinated and 5.0% of the control group became ill. Protective efficacy was 74.0% in the whole material and 66.1% in the patients older than 1 month (Table I and Fig. 2).

Table I
Protective effect of vaccination

Age	No. of infants		<i>E. coli</i> enteritis				Protective efficacy, per cent
			No. of cases		per cent		
	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	
<i>0-month old</i>							
1. Immature (< 2500 g)	223	304	17	33	7.6	10.9	30.3
2. Mature (> 2500 g)	104	214	5	15	4.8	7.0	31.4
Total	327	518	22	48	6.6	9.5	30.5
<i>Above 1 month</i>							
3. 1—2 months old	71	118	3	11	4.2	9.3	54.8
4. Above 2 months	229	404	3	20	1.3	5.0	74.0
Total	300	522	6	31	2.0	5.9	66.1

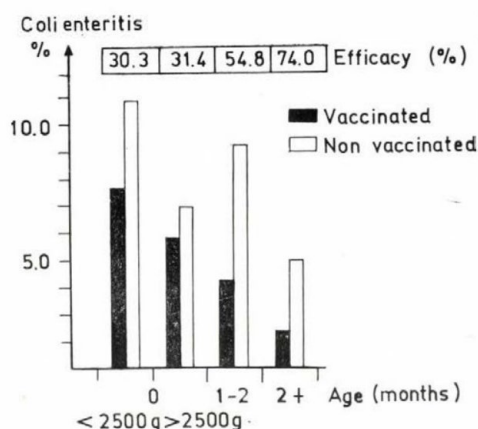


Fig. 2. Efficacy of vaccination. *E. coli* enteritis in vaccinated and non-vaccinated groups

Effect of immunization in prolonging the onset of E. coli enteritis. With a few exceptions, *E. coli* infections among the immunized and non-immunized infants over 1 month of age occurred in the first month of stay in the hospital.

In the 0-month-old group there was a significant difference between the immunized and non-immunized patients. In the latter group, 72.9% fell ill during the first month of hospital stay, while 25.0% in the second, and 2.1% in the subsequent months. In the immunized group the situation was reversed, with only 31.8% during the first month of hospital stay, 50.0% during the

Table II

Effect of immunization in prolonging the onset of E. coli enteritis

Period between hospital admission and onset of enteritis	E. coli enteritis							
	No. of cases				per cent			
	0 month		> 1 month		0 month		> 1 month	
	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control
Less than 30 days	7	35	6	25	31.8	72.9	100.0	80.6
30-60 days	11	12	—	4	50.0	25.0	—	12.9
More than 60 days	4	1	—	2	18.2	2.1	—	6.5
Total	22	48	6	31	100.0	100.0	100.0	100.0

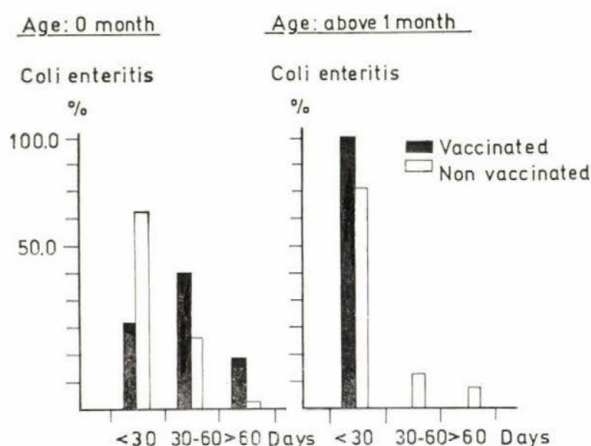


Fig. 3. Period between admission and onset of disease

second, and 18.2% in the later months. Thus, vaccination postponed the onset of disease. In the immunized 0-month-old group it was these late infections that accounted for the higher morbidity of the patients and the lower protective effect of the vaccine (Table II and Fig. 3).

A possible explanation of the above observation is that the effect of immunization seems more lasting when made above 1 month of age, and, presumably due to immunobiological factors, immunity of the infants less than 1 month of age rapidly decreases from the second month following basic immunization. Consequently, part of the infants living in an infected environment will contract the disease.

Effect of immunization on the clinical course of E. coli enteritis. In both the immunized and the non-immunized groups, 40% of the patients had ente-

ritis of moderate severity, 30% a mild, and 30% a severe form of the disease. Symptom-free carriers amounted to 3.5% in both groups. Thus, vaccination had no effect on the course of the disease.

Oral vaccination of infants against enteropathogenic *E. coli* infections seems suited for the prevention of the disease especially in hospitals, day-nurseries or other infant institutions where the disease is common. The vaccination had no untoward effect, its administration was simple.

Immunization of infants older than 1 month had a good efficacy, the risk of contracting the disease could be reduced to one third.

Results were less satisfactory in the vaccinated 0-month group, due to late infections. Immunity was of a sufficient degree in the first months, then rapidly decreased and the infant living in an infected environment contracted the disease. In order to make immunity more lasting it is suggested that basic immunization should be repeated at 2-week intervals and the dosage should be increased.

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ORAL IMMUNIZATION WITH AN EXTRACT OF ESCHERICHIA COLI ENTERITIDIS

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Summary. In the period from 1st August, 1970 to 20th November, 1972, a total of 6255 children was vaccinated with extract vaccine and given a booster, as against a group of 12 870 children who did not receive vaccination and booster. Among the vaccinated and boosted children, 1 child contracted an *Escherichia coli* O55 infection, while among the non-boostered children 6 contracted such an infection. Among the vaccinated and boosted children there was one case, while among the non-boostered children there were 9 cases of *E. coli* O111 infection.

Escherichia coli enteritis is predominantly a disease of early childhood. After infection, young infants fall ill more frequently and also more seriously than older ones. This is obviously due to an immunity formed suddenly or developing gradually. Thus the question arose whether by feeding presumably harmless *E. coli* antigens it is possible to present the mucosa of the newborn's intestine with the same immunological experience as possessed by older children and adults. With this purpose we developed a sodium desoxycholate extract of *E. coli*. It was carefully tested serologically as well as in animal experiments and, after assessing its harmlessness in adult volunteers it was applied in field trials to test it clinically. The present paper will report on some results of these field trials.

In the GDR, nearly 99% of all babies are delivered in maternity wards where they stay for 6 days. During this period we perform a basic immunization of each newborn baby with 2 ml each of an extract vaccine of the antigenic components of *E. coli* O111 and O55. The vaccine is given orally with a syringe (Fig. 1), starting on the second day of life. After 4—6 weeks the infants are presented at the infant welfare centre. At that time a total immunizing dose of 10 ml is fed as a booster (Fig. 2). Certain difficulties are connected with such a vaccination. Some infants spit out the vaccine. Large quantities such as 10 ml can hardly be administered without loss. When the vaccine was given together with mother's milk the speed of drinking increased. Such a combination, however, is not feasible in mass vaccinations. Therefore we tested other additions of 5% and 10% glucose solution. The number of sucking movements and the period for sucking 10 ml of the test solution were taken as the criterion of the speed of drinking as described by KRON *et al.* [3]. Fourteen in-



Fig. 1. Application of extract vaccine in newborns



Fig. 2. Application of booster dose

fants drank the vaccine mixed with 10% glucose solution in 175 s with 29 sucking movements per min; 49 infants drank the vaccine mixed with mother's milk in 94 s at a rate of 39 sucking movements per min; 50 infants drank the vaccine mixed with 5% glucose in 56 s at a rate of 46 sucking movements. We feel that this procedure is adequate to be taken as a "consumer's criterion" for oral vaccines in this age group.

Our method of vaccination has been tested in a large field trial in the Schwerin district for several years. From the point of organization, the vaccination was found to be feasible. About 90% of all the newborns were involved in the immunization process with boosting. As the vaccination was made on a voluntary basis (the mothers were asked to consent to it) we consider this

a good result. The vaccination proved harmless and was tolerated well. No side reactions have been observed. The question of efficacy cannot yet be answered reliably. Since the introduction of immunization, no epidemic of *E. coli* enteritis has been noted in the particular district, thus exposure is not high. As to the results so far achieved, the figures were obtained by continuously comparing the cases recorded by the local Medical Officers of Health with the names of the immunized and non-immunized children. They were controlled by the *E. coli* enteritis registers of the laboratories. A case analysis was performed in each instance to ensure that only cases with a reliable diagnosis were evaluated (Table I).

Table I
Preliminary results of field trial in the Schwerin district

	No.	Cases of enteritis		
		O111	O55	Total
Vaccinated and boosted children	6255	1	2	3
Non-boostered children	12 870	9	6	15
Correlation		1 : 4.5	1 : 3	1 : 3.75

The results were as follows. In the period from August 1st, 1970 to November 20th, 1972, 6255 babies were vaccinated with extract vaccine and given a booster as against a group of 12 870 babies who did not receive vaccination or a booster. Among the vaccinated and boosted babies, one contracted an *E. coli* O55 infection, while among the non-boostered babies six contracted such an infection. This results in a ratio of 1 : 3. Among the vaccinated and boosted babies there was one case of *E. coli* enteritis O111, and among the non-boostered, nine cases of *E. coli* O111 infection occurred. This means a ratio of 1 : 4.5. Thus, the net result is 2 cases in the booster group and 15 cases among the non-boostered. Comparing the boosted babies with the non-boostered ones, the ratio is 1 : 3.75.

In a period without *E. coli* enteritis epidemics it is difficult to obtain evidence of the vaccination's efficacy. However, the statistics seemed to point to the effectiveness of the vaccine. The figures so far obtained may in any case serve to justify further trials of oral immunization with extract vaccine.

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A SENSITIVE AND RAPID METHOD FOR ANTIBODY DETERMINATION BY GEL ADSORPTION

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Summary. Bacterial strains belonging to the *Enterobacteriaceae* family and exhibiting the same serotype may differ in $\text{Al}(\text{OH})_3$ gel adsorption. The differences in adsorption offer a possibility for intratypical classification. The adsorption capacity of bacteria exhibiting weak adsorption increases if homologous antibodies bind to their surface. This phenomenon allowed to develop a sensitive method for the determination of antibodies. Labelling with ^{32}P of the test bacterium (antigen) increases the sensitivity of the method and facilitates reading of the results. Several experiments have proved that the method indicates the presence of specific antibodies and is by about $3 \log_{10}$ exponents more sensitive than the classical procedure. The method was used for the determination of antibody titres against pathogenic *Escherichia coli* in the blood of women in labour and in umbilical blood samples taken during labour. Similarly, antibody titres were also determined in mother's milk and in the faecal suspension of infants fed with mother's milk. Data obtained during antibody titration in children vaccinated orally against dysentery are presented.

It has been reported earlier that the $\text{Al}(\text{OH})_3$ gel adsorption of strains belonging to the family *Enterobacteriaceae* show intratypical differences making it possible to separate strains of the same sero or phage types [1].

The method is based on inhibition of adsorption by secondary phosphate ions. Thus the bacterial cells are added to the gel at increasing phosphate buffer concentrations and that molar concentration of the buffer is determined at which adsorption of 50% of the bacteria is inhibited. The numerical value of this molarity (AC_{50} value) is characteristic of a given bacterial strain.

In the *Escherichia* group, the strains qualified as pathogenic on the basis of their serotype have the common property of binding weakly to gel. Among other pathogenic enteric bacteria within the same serotypes strains with weak and relatively strong adsorption capacity could be isolated.

Changes occurring on the surface of bacteria may influence their adsorption capacity if they affect properties involved in adsorption. Binding of homologous antibodies to the surface may alter the adsorption capacity of the cell and thus allow for a sensitive and rapid detection or a quantitative determination of antibodies in a suitable experimental design [2].

For this determination, within each serotype a test strain characterized by a low AC_{50} value is required since its adsorption is increased by binding antibodies. On this basis, the presence of homologous antibodies may be demonstrated with high sensitivity.

In the present experiments, the possibilities of the detection mainly of enteric bacteria and of the antibodies acting against them have been studied and of the results, the practical application of the gel adsorption method for the determination of antibodies is reported within the *E. coli* and the *Shigella flexneri* groups.

As a culture medium, 20 ml meat broth (pH 7.5) in Erlenmeyer flasks was used. For a radioactive labelling of the cultures aliquots of 0.25 mCi $\text{Na}_2\text{H}^{32}\text{PO}_4$ were added to the cultures.

Buffer solutions. Of a 0.66 M phosphate buffer pH 7.5 stock solution, dilution series were prepared under 0.1 M with Tris-HCl buffer 0.05 M, pH 7.5 and above 0.1 M with deionized water. As dilution fluid, physiological saline stabilized with 20% Tris buffer was used. The adsorbent was $\text{Al}(\text{OH})_3\text{C}\gamma$ (Behring Werke). The suspension contained $\text{Al}(\text{OH})_3$ in a ratio corresponding to 1 g Al_2O_3 /100 ml deionized water. Before use, the suspension was diluted 1 : 4 with deionized water.

Experimental material. Blood obtained from pregnant women, umbilical cord blood taken at birth, mother's milk, and faecal extracts of children immunized against *Shigella* by oral vaccination.

Preparation of bacterial cell suspensions was done as follows. Aliquots of 0.25 mCi $\text{Na}_2\text{H}^{32}\text{PO}_4$ were measured into flasks containing 20 ml broth and inoculated with loop quantities of the *E. coli* strain chosen for study. The flasks were incubated in shaking water bath of 37°C for 4 hr or overnight. The cells were centrifuged and the pellet was washed 3–4 times with a quantity of washing fluid equal to the initial volume or washing was continued until activity was negligible as compared to that of the cell suspension resuspended in the initial volume.

Studies on absorption properties. The principle of the procedure is that adsorption to $\text{Al}(\text{OH})_3$ gel is inhibited by secondary phosphate ions and thus the rate of adsorption can be expressed by the molarity of phosphate buffer inhibiting the adsorption of 50% of the cells. Aliquots of 1 ml aluminium hydroxide gel are measured into 8 test tubes (100 × 15 mm), while one test tube contains 1 ml of deionized water. Then 2 ml Tris buffer is measured into the first tube and 2 ml phosphate buffer from each dilution of a $\log_2$ series is added to the rest of the tubes. The highest and the lowest dilutions of the buffer are 0.005 and 0.32 M, respectively. Finally, aliquots of 1 ml of the labelled bacterial suspension (diluted to 10 000 cpm/ml) are added to the tubes. The tubes are then mixed by shaking and incubated at room temperature for 15 min. After centrifugation at 1000–2000 rpm for 1 min, the supernatant's radioactivity is determined. The activity found at 0.32 M phosphate buffer corresponds to that of the control without gel since at this concentration bacterial cells fail to adsorb to the phosphate buffer. Thus the activity measured in the supernatant at 0.32 M phosphate buffer is considered 100% and the activities found

at other molarities are expressed in percentage of the former. To characterize the bacterial strains, the numerical value of that molarity of the phosphate buffer is used at which — compared to the final concentration — half of the cells is in the supernatant (AC_{50} value). The result of such an experiment is shown in Fig. 1.

It can be read from Fig. 1 that the bacteria studied adsorb to the gel at a 50% ratio at 0.073 M phosphate buffer, thus, the adsorption coefficient of the strain is 0.073.

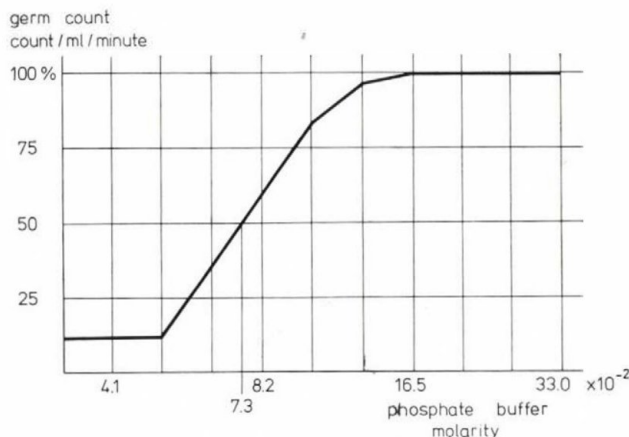


Fig. 1. $Al(OH)_3$ gel adsorption of *E. coli* strain

In the absence of phosphate ions, 70–80% or less bacteria of the *E. coli* enteritis group adsorb to the gel. Up to now, of all the strains studied only serotype O124 behaved in a different way.

Titration of antibodies against *E. coli*. From the antibody containing material, a two-step dilution series is prepared. The last tube contains only dilution fluid (control). Aliquots of the labelled bacterial suspension are then given to the tubes; after incubation at 37°C for 3 hr, the adsorbent is added and incubation is repeated at room temperature for 20 min. After centrifugation at low speed, activity of the supernatants is determined.

The antibody titre of the serum corresponds to that dilution at which the activity (cell count) of the supernatant is 50% of that of the control tube. If the antibody titre is to be determined with high accuracy, the values obtained are compared to a test performed with standard serum.

Optimum titration parameters. Absorbent. An arithmetical dilution series from 1 : 1 to 1 : 10 was prepared with 1% $Al(OH)_3$ gel. Aliquots of a bacterial suspension with known adsorption properties were added in the presence of phosphate buffer corresponding to AC_{50} , and the dependence of adsorption on

gel dilution was studied. It was found that adsorption showed no essential change up to a gel dilution of 1 : 6. For antibody determination, 1 ml aliquots of a 1 : 4 gel dilution were used.

Optimum ionic concentration of phosphate. In order to determine the ionic concentration of phosphate in the medium yielding the highest antibody titre, from *E. coli* O55 serum (agglutination titre 1 : 16–1 : 32), 3 parallel log₂ dilution series were prepared in 0.5 ml dilution fluid. From a [³²P] *E. coli* O55

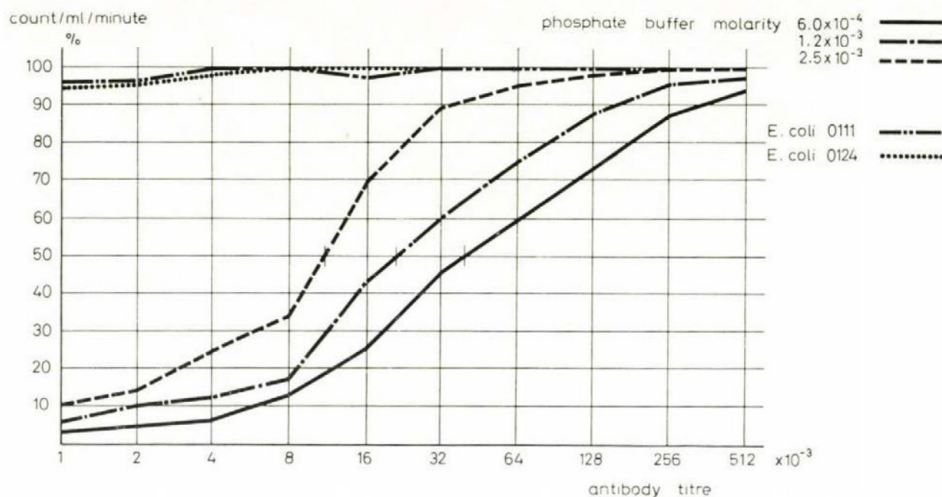


Fig. 2. Correlation between phosphate buffer molarity and antibody titre of medium

suspension corresponding to 10 000 cpm/ml, aliquots of 0.5 ml were added to the dilutions and they were incubated in a water bath at 37°C for 3 hr. Then 0.1 ml aliquots of phosphate buffer were added to the antibody dilutions so that the final dilution of the phosphate buffer was 2.5×10^{-3} , 1.2×10^{-3} , and 6.0×10^{-4} M in the first, second, and third dilutions, respectively. Aliquots of 1 ml of a 1 : 4 dilution of the absorbent were then added. Incubation at room temperature for 20 min was followed by a centrifugation at 2000 rpm for 1 min and activity of the supernatant was determined. The same experiment was carried out with *E. coli* O111 and O124 strains in order to obtain control data. Titre values are shown in Fig. 2.

As shown in Fig. 2, phosphate buffer molarity in the medium was inversely related to the antibody titre. With *E. coli* O111 and O124 as antigens, no antibody titre were obtained.

Optimum cell count added to the serum dilution. Of the serum sample used for diagnostic purposes (agglutination titre 1 : 16), 6 parallel log₂ dilution series were prepared. The medium contained no phosphate ions in 3 of the parallels

while the final ionic phosphate molarity was 1.2×10^{-3} in the other 3 parallels. The quantity of bacterial cells varied as follows. Aliquots of 0.5 ml from cell suspensions corresponding to 10 000, 5000, and 2500 cpm/ml were added to each dilution series. After incubation at 37°C for 3 hr, 1 ml aliquots of the absorbent were added. After incubation at room temperature for 20 min and slow centrifugation, activity was determined in the supernatant. The titres are shown in Fig. 3.

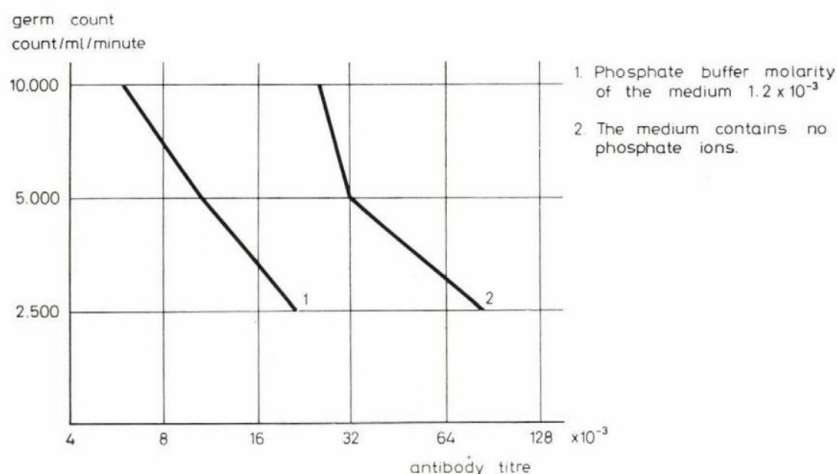


Fig. 3. Correlation between germ count and antibody titre

Fig. 3 clearly shows the inverse relation of antibody titre with phosphate ion concentration or germ count. The highest titre was obtained at the lowest germ count in a phosphate free medium. In further experiments a germ count corresponding to 5000 cpm/ml (about 2.5×10^7 germs) was used. Sensitivity of the gel adsorption technique as an indicator of the presence of antibodies was found to be about $3 \log_{10}$ exponents higher than that of the classical agglutination test.

The question of the materials influencing the results of titrations seemed interesting, as they might affect the titration of antibodies. Results obtained with various materials studied in acid and neutral media are summarized in Table I.

Under the present experimental conditions mainly serum proteins and materials present in the faecal extracts might have had a significance as inhibitors of cellular adsorption, especially in the case of low antibody titres. Therefore, for studying such materials, a centrifugation after incubation at 37°C and a washing of the bacterium-antibody complex with one or two changes of physiological saline were done. In this way, disturbing materials

Table I
*Materials influencing adsorption to $Al(OH)_3$ gel of *E. coli**

Material	pH	Concentration	Efficacy
Na_2HPO_4	5.6	0.1 M	—
Na_2HPO_4	7.0	0.1 M	+
NaCl	7.5	1.0 M	—
NaCl	9.0	1.0 M	—
$NaHCO_3$	8.5	0.1 M	—
$(NH_4)_2SO_4$	7.5	0.1 M	—
CH_3COONa	7.5	0.5 M	—
Tris-HCl	7.5	0.5 M	—
Oxalic acid	7.5	0.1 M	—
Malonic acid	5.5	0.2 M	—
Malonic acid	7.5	0.2 M	+
Maleic acid	5.5	0.2 M	—
Maleic acid	8.0	0.2 M	—
Fumaric acid	7.5	0.2 M	—
Pyruvic acid	5.0	0.2 M	—
Pyruvic acid	7.5	0.2 M	+
Citric acid	5.5	0.2 M	—
Citric acid	7.5	0.2 M	+
Dextran sulphate	5.5	1%	+
Dextran sulphate	7.0	1%	+
Human serum	5.5	1%	±
Human serum	6.0	1%	+
Human serum	7.0	1%	+
Human serum	8.0	1%	+
Faecal extract	5.5	1%	+
Faecal extract	7.0	1%	+
Faecal extract	8.0	1%	+

For adjusting the pH, Tris-HCl or maleic acid buffer was used

were removed from the reaction mixture. Further determination was the same as above.

It was important to clarify how heat inactivation influenced the titres obtained by the gel adsorption method of antibody determination. For this purpose, mother's milk defatted by centrifugation was incubated at various temperatures for 30 min and then *E. coli* O55 and O111 antibody titrations were carried out. Results are shown in Table II. The results suggested that the

Table II

Antibody titres of mother's milk against pathogenic E. coli O55 after heat inactivation

Incubation temperature, °C	Antibody titre	
	<i>E. coli</i> O55	<i>E. coli</i> O111
20	128	512–1024
60	128	—
65	—	256–512
70	—	16–32
75	—	<2
80	<2	<2
100	<2	<2

changes found in the titres corresponded to those characteristic of heat inactivation of antibodies. This means that the gel adsorption method fails to indicate the presence of heat-inactivated antibodies.

In order to obtain data on the specificity of the method, samples of mother's milk absorbed by *E. coli* O55 and O111 were studied. Broth cultures of *E. coli* O55 and O111 were washed with physiological saline several times and then the pellets were incubated with aliquots of the same sample of mother's milk at 37°C for 3 hr. After centrifugation, the antibody titres against *E. coli* O55 and O111 were determined in the supernatant (Table III).

The results suggested that the titre drops after depletion by bacteria of the same serotype, but it remains unchanged when tested against heterologous strains.

A similar experiment was carried out with human serum absorbed by bacteria. For absorption, *E. coli* O55 or *Shigella dysenteriae* 2 was used. The antibody titres of the absorbed sera were determined against *E. coli* O55 and

Table III

Antibody titre of mother's milk absorbed with E. coli

Strain used as antigen	Titre of milk sample before absorption	
<i>E. coli</i> O55	1024–2048	
<i>E. coli</i> O111	512–1024	
	Titre of milk sample after absorption	
	<i>E. coli</i> O55	<i>E. coli</i> O111
<i>E. coli</i> O55	<2	2048
<i>E. coli</i> O111	512–1024	<1

Table IV
Antibody contents of human sera absorbed by E. coli O55 or S. dysenteriae 2

Strain used as antigen	Reciprocal serum titre		
	before absorption	after absorption	
		<i>E. coli</i> O55	<i>S. dysenteriae</i> 2
<i>E. coli</i> O55	2.56×10^4	$< 4 \times 10^2$	$1.28 \times 10^4 - 2.56 \times 10^4$
<i>S. flexneri</i> 3a	$5.12 \times 10^4 - 1.02 \times 10^5$	$5.12 \times 10^4 - 1.02 \times 10^5$	1.02×10^5

S. flexneri 3a strains. The data obtained are shown in Table IV. The data indicated that the antibody titres against *S. flexneri* were unchanged after depletion with either *E. coli* O55 or *S. dysenteriae* 2 cells.

In order to obtain more data on the specificity of the method, a further model experiment was performed. Thin layers of broth cultures of *E. coli* O55 were irradiated with UV-light until no colony formation was observed after plating loopful amounts of the irradiated cultures on eosin methylene blue plates. Of this partially inactivated cell suspension, a single dose of 1 ml was injected into the ear-vein of a rabbit. Antibody titres were determined in various blood samples taken before and after injection using *E. coli* O55 and O111 strains as antigen. The results of this experiment are shown in Table V. The data showed an increase of the antibody level corresponding to *E. coli* O55 used for immunization. The difference in the sensitivity of the agglutination and the gel adsorption methods was again of a magnitude of $3 \log_{10}$.

Using *S. flexneri* strain 3a as antigen, the antibody level was determined in some frequently studied materials in order to obtain information as to what titres could be expected to occur with their use. Results are shown in Table VI. The agglutination titre of the serum used for diagnostic purposes was 1 : 32–1 : 64, viz. the sensitivity of the method was about $3 \log_{10}$ in this case, too. The high titre of normal rabbit serum might be attributed to the effect of a common antigenic component. It is known e.g. that enterococci common in rabbits contain shigella antigenic components. Similar titres were found in the sera from two healthy persons while about a 30 times higher titre was estimated in the serum of an orally vaccinated child.

Table V
Antibody titres of serum against E. coli O55 and O111 in rabbits immunized with E. coli O55

Strain used as antigen	Reciprocal serum titre before immunization	Reciprocal serum titre after immunization	
		at 6 days	at 19 days
<i>E. coli</i> O55	$< 10^2$	$2.048 \times 10^6^*$	$2.048 \times 10^6^*$
<i>E. coli</i> O111	$< 10^2$	10^2	10^2

* Reciprocal agglutination titre, 8×10^2

Table VI
Titration of S. flexneri antibodies by gel adsorption method

Sera	Reciprocal antibody titre Test strain: <i>Shigella flexneri</i> 3a
<i>S. flexneri</i> polyvalent	3.2×10^4
<i>S. flexneri</i> typing	1.6×10^3 – 3.2×10^3
<i>E. coli</i> O55	$< 10^2$
Normal rabbit	2.04×10^3 – 4.09×10^3
Healthy subject No. 1	2.04×10^3 – 4.09×10^3
Healthy subject No. 2	4.09×10^3
Patients of children's home, vaccinated against dysentery	6.4×10^4 – 1.28×10^5

Subsequently, the method was used for screening in two communities. In one of the screening studies, the *E. coli* O55 antibody content was estimated in blood samples taken from women before labour, cord blood samples taken during labour, milk samples taken from the same women during labour and of the stools of their breast-fed infants. The data obtained in these studies are summarized in Table VII. In these materials measurable titres were obtained, though the titres for the corresponding materials showed no correlation. In

Table VII

Antibody contents against E. coli O55 in samples of blood and milk of women in labour and in faecal samples of infants fed with mother's milk

Serial number	Persons studied	Reciprocal antibody titre against <i>E. coli</i> O55			
		blood	cord blood	mother's milk	infant's faeces*
1	T. M.	2048	1024	128–256	256
2	B. J.	2048	64–128	128–256	16–32
3	Ö. F.	2048	1024	2048	—
4	T. F.	2048	1024	1024–2048	256
5	D. L.	2048	1024	512–1024	32–64
6	Sz. L.	1024–2048	512–1024	—	—
7	Sz. F.	1024–2048	16–32	1024	16–32
8	B. V.	1024	64	1024–2048	64
9	M. G.	512–1024	64–128	—	—
10	B. S.	128–256	32–64	1024–2048	16–32
11	K. T.	10	10	8	< 2

* Starting dilution, 1 : 5

case where the titre in the maternal serum was low, it was also low in the other corresponding materials.

Another screening study was made in a children's home. During this study the blood and copro-antibody titres were determined in children vaccinated against dysentery. Part of the patients received systematic vaccination from July 7, 1969 to December 30, 1972. The vaccine had been produced from *S. flexneri* strains and supplemented with *Shigella sonnei*. As controls, the materials of unvaccinated children were studied. The serum samples were taken about one week after feeding the pills. Faecal samples were collected about 3 months after vaccination. An oral booster dose was given to 8 patients from whom further faecal samples were collected one week later. The antibody titres were determined as described above; as test bacterium (antigen), *S. flexneri* strain 3a was used which had been included in the vaccine throughout. Results are shown in Table VIII.

High antibody titres were found in the children's home especially in the vaccinated group, as compared to those found in the two healthy subjects shown in Table V. Copro-antibodies occurred more frequently in the immunized children but the small number of cases studied allows no conclusions. In two faecal samples collected after the booster vaccination, no copro-antibodies could be detected. As to the distribution of dysentery negative and positive cases in the vaccinated and control groups, patients who showed characteristic symptoms of dysentery and from whom *S. flexneri* was isolated were classified into the dysentery positive group. The patients belonging to the dysentery negative group also showed symptoms, but no bacteria could be isolated from them. This classification is shown in Table IX. The data show a random distribution and fail to reflect any protecting effect of vaccination.

The most reliable way for estimating the efficacy of vaccination undoubtedly consists of epidemiological observations on a high number of vaccinated subjects and a statistical evaluation of the data obtained. For assessing of individual immunity, a well-chosen *in vivo* test such as the detection of antibodies is the most reliable. Nevertheless, *in vitro* serological tests may also be useful especially if the relation between the serological tests and the immunization experiment is known. For the time being, our work has been aimed at the development of a rapid and sensitive method of antibody detection. Our results must, however, be completed by comparisons with immunization experiments *in vivo*.

The advantage of our method is its rapidity and its sensitivity which surpasses most serological procedures. The model experiments convincingly showed that the procedure indicates homologous antibodies. Since radioactive bacteria are used, circumstantial germ counting is avoided. Bacterial contamination of the materials exerts no disturbing effect on the results (e.g. at the determination of copro-antibodies).

Table VIII

Serum and copro-antibody titres in children orally vaccinated against dysentery and in the control group

Serial number	Treatment	Persons studied	Reciprocal antibody titre, test strain <i>S. flexneri</i> 3a		
			Serum × 1000	Copro-antibody*	
				3 months after immunization	1 week after booster
1	Non-vaccinated	Sz. K.	32-64	—	—
2		K. N.	16-32	<6	—
3		Sz. A.	16-32	—	—
4		K. N.	—	<6	—
5		V. S.	—	<6	—
6		K. S.	64	<6	—
7		M. J.	—	<6	—
8		M. K.	16-32	—	—
9		J. T.	64-128	12	—
10		H. E.	64-128	<6	—
11	Vaccinated	G. I.	64-128	6-12	—
12		Sz. A.	64	6-12	24
13		K. J.	—	6-12	12
14		G. K.	64-128	—	—
15		F. J.	128-256	—	—
16		T. E.	16-32	—	—
17		S. S.	64-128	—	—
18		R. H.	—	<6	—
19		G. L.	—	<6	48-96
20		F. J.	128-256	—	<6
21		G. E.	512	—	—
22		G. É.	128	—	6
23		B. S.	512	—	6
24		N. A.	16-32	—	<6
25		B. M.	256-512	—	—
26		C. E.	—	—	24

* Starting dilution, 1 : 5

Due to the small number of cases included, the results of the two screening studies are not suitable for drawing conclusions. The data obtained suggest, however, that well-measurable antibody titres can be detected in the samples and the method may provide useful data for the assessment of the efficacy of vaccination during campaigns. The observation that a mucous diarrhoea

Table IX
Distribution of vaccinated and non-vaccinated children

Time of examination	No. of reported cases	Dysentery positive		Dysentery negative	
		vaccinated	non-vaccinated	vaccinated	non-vaccinated
1969	12	5	1	2	4
1970	34	22	10	—	2
1971	22	3	5	8	6
1972	12	5	2	2	3
Total	80	35	18	12	15

without fever occurred frequently on the day of and one day after vaccination, deserves attention. A transient stomatitis after vaccination has also been reported. All these direct attention to allergic phenomena which may confuse a classification on the basis of clinical symptoms. It is also conspicuous that in the children's home high antibody titres were found even in the control group. It must, however, be considered that the vaccinated and the control groups do not live in strictly closed communities and there are nosocomial strains circulating in institutions. Many of the children are mentally retarded and coprophagy is not uncommon. Thus, the oral vaccination was not alone responsible for their immunization.

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HUMAN IMMUNIZATION WITH KILLED CHOLERA VACCINE

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Summary. Oral immunization of 50 human volunteers and reimmunization of a 2nd group of 50 subjects was carried out with killed cholera vaccine in pill form. The dose of antigen in one pill was equal to 120×10^9 vibrios of the classical Inaba and Ogawa and El Tor strains. The vaccine was applied by taking one pill in the morning 60 min before breakfast on 5 consecutive days. The reimmunized individuals had been immunized subcutaneously 6 to 12 months earlier. The titres of vibriocidal antibodies and agglutinins in the serum, and of vibriocidal antibodies in the faeces were studied from the 7th to the 42nd day after immunization. As a result of the immunization the primary immune response developed with the appearance of vibriocidal antibodies in the serum, without establishing vibriocides in the intestinal contents. The primary humoral immune response was weak. In approximately 70% of the immunized subjects the titre varied in the range from 1 : 10 to 1 : 320. Seroconversion was significant statistically. After reimmunization, a prolonged negative phase occurred in the immune response, with a maximum between the 7th and 14th days. The negative phase was followed by an increase of serum antibodies from the 21st to the 28th day after which a new decrease occurred.

The aim of the present study was to trace the course of the humoral and local immune response in a group of volunteers immunized and reimmunized with killed cholera vaccine.

Fifty volunteers were immunized orally with cholera vaccine, and fifty other subjects, who during 1971—1972 had been immunized subcutaneously with 2 or 3 injections, were reimmunized orally after 6—12 months.

Vaccine was produced from 4 strains: classical Ogawa and Inaba and El Tor Inaba and Ogawa. Every pill contained antigen equal to 120×10^9 killed vibrios. One pill was taken every morning 60 min before breakfast on 5 consecutive days.

The titres of the vibriocidal antibodies and haemagglutinins in the serum were estimated prior to immunization and on the 7th, 14th, 21st, 28th and 42nd days after immunization, and the copro-antibodies on the 5th, 7th, 9th, 12th, 14th, 21st and 28th days.

It is seen from Fig. 1 that primary immunization with killed vaccine led to a definite but usually weak seroconversion. The vibriocidal antibody titre increased up to the 21st day by 2—3 degrees over the initial level in 70% of the immunized individuals. The titres obtained were not high, as absolute values moved in the range from 1 : 10 to 1 : 320 (mean 1 : 80), with the highest level on the 14th day. The agglutinin titres were still lower and non-

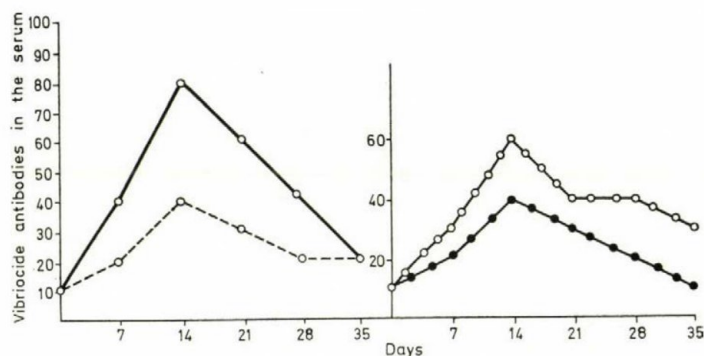


Fig. 1. Immunization of humans with killed cholera vaccine. — *V. cholerae* Ogawa; — — *V. cholerae* var. *el-tor* Ogawa; ○-○- *V. cholerae* Inaba; ●-●- *V. cholerae* var. *el-tor* Inaba

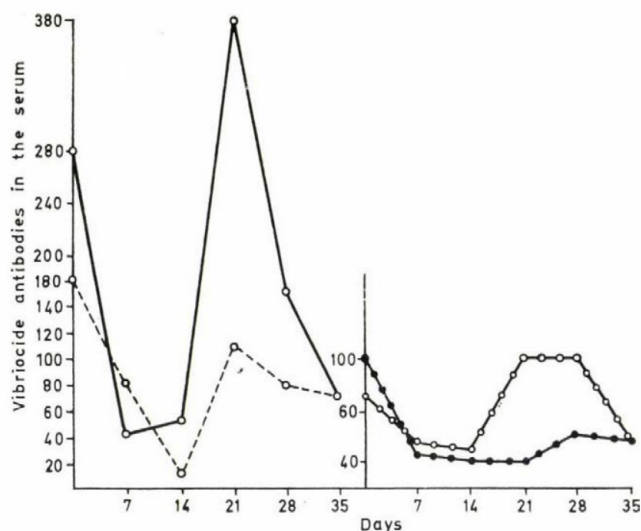


Fig. 2. Oral reimmunization of humans with killed cholera vaccine. — *V. cholerae* Ogawa; — — *V. cholerae* var. *el-tor* Ogawa; ○-○- *V. cholerae* Inaba; ●-●- *V. cholerae* var. *el-tor* Inaba

significant statistically and this was the case with the antibody titres against all the four strains included in the vaccine.

Study of the faeces from the 5th to the 21st day for the presence of vibriocidal and haemagglutinating antibodies by means of passive haemagglutination gave negative results.

It is seen from Fig. 2 that oral reimmunization of subcutaneously immunized persons led to characteristic changes in the serum vibriocidal antibody contents. A sharp drop of the antibody titre by 2 to 5 degrees was observed

on the 7th and 14th days which continued up to the 21st day for the El Tor Inaba strain. On the 21st day there followed a complete restoration of the vibriocidal antibody titre to the classical Ogawa and Inaba strains, the titre against the Ogawa strain reaching a higher than the initial level. At the same time there was a rise in the titre to the El Tor Ogawa and Inaba strain but without reaching the initial level. On the 28th day the titre dropped again. Similar dynamics were observed both in the subjects who prior to the immunization had had high and medium initial titres in the order of 1 : 160 to 1 : 2560 and in those with lower titres. In subjects displaying high titres on the 21st day the blood antibody content was restored to or near to the initial level, while in those with low vibriocidal antibody titres to a level equal to, or one or two degrees above, the initial titre. The agglutinins generally showed low values; a considerable drop having occurred up to the 21st to 28th days. However, it was not possible to make independent conclusions without considering the changes in vibriocidal antibody content.

Study of the faeces for vibriocidal antibodies gave a negative result. In a single subject did the vibriocidal titre against the four strains attain 1 : 10 on the 8th day after reimmunization. Copro-antibodies could not be established by means of passive haemagglutination.

The presence of a negative phase, *i.e.*, a drop of the titres from the 7th to the 21st days, showed that after oral immunization a large amount of antigen enters the blood, and is bound there by the antibodies. Then a phase of immunoclearance of the antigen follows as a result of which the antibodies remain at a low level for a long time. Meanwhile, as a result of the new antigenic stimulus, the rise of the titres is delayed to after the 14th day, with a peak on the 21st day. Still, this process of antibody synthesis and secretion does not cause a considerable increase of the vibriocidal antibodies to titres significantly higher than those observed before reimmunization.

In this context, two questions arise: (i) why is it that in the presence of a sufficient quantity of antigen, as manifested by the negative phase, after oral immunization the secondary synthesis of antibodies failed to result in the higher indices characteristic of parenteral immunization; and (ii) whether immunoclearance caused a change of the immunochemical class of the synthesized antibodies, *i.e.* was only IgG synthesized or had the synthesis of IgM continued? And did the newly synthesized antibodies differ in affinity and vibriolytic efficiency from those produced after the first and second subcutaneous injection?

At titration, there were remarkable differences in vibriolysis between the various sera. Thus, in one instance with the same vibriocidal antibody titre of 1 : 640, the boundary line between the clear and the turbid test tubes was clearly outlined. Clear test tubes are indicative of a complete lysis while turbidity points to an intensive growth of cholera vibrios. With other sera

the boundary was less sharp and in 2—3 or sometimes more test tubes the fluid was half-clear, indicating an incomplete vibriolytic effect. For example, in one case the 1 : 640 test tube was absolutely clear and the 1 : 1280 one absolutely turbid, while with another serum we observed half-turbid test tubes in the range from 1 : 80 to 1 : 640. This might refer to differences in the immunochemical behaviour of IgG and IgM. These are questions which must be elucidated before a correct answer can be given as to the significance of humoral immunity and of the different kinds of antibody in immunity against cholera.

Oral immunization with cholera antigen corresponding to 120×10^9 cells led to a primary immune response with appearance of vibriocidal antibodies in the serum of the immunized subject, without the development of a local immune response with the appearance of vibriocidal antibodies in the faeces.

The primary immune response was comparatively weak or of medium intensity in about 70% of the immunized subjects. The antibody titre against the various strains varied from 1 : 10 to 1 : 320 (mean 1 : 60). The seroconversion observed was significant statistically.

In parenterally immunized subjects on oral reimmunization 6 to 12 months later with killed cholera vaccine, a negative phase occurs between the 7th and 14th days, continuing sometimes even after the 21st day. The negative phase is followed by an increase in serum antibodies, which may reach and surpass the initial level.

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A NEW INACTIVATED ORAL CHOLERA VACCINE

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Summary. A technology has been elaborated for producing an inactivated cholera vaccine for oral administration. The vaccine showed a good compatibility in human volunteers. The results of laboratory controls are reported.

Since the beginning of the seventh pandemic in 1961 cholera has once again emerged as an international health problem. During the last years WHO has offered international surveillance programmes [1–4] to prevent spread of the disease. Although the improvement of sanitation and hygiene is a dominant factor in limiting intestinal infections, vaccination methods have also been discussed [5–8]. Conventional parenteral vaccines provide protection to about 50% of vaccinated subjects [9, 10]. As this protection lasted for a few months only, endangered persons were revaccinated every sixth month. The contraindications and the increase of hypersensitivity reactions are limiting factors of such vaccination procedures while oral immunization methods are free from these disadvantages. The safe and easy way of administering an inactivated vaccine in tablet-form offers the possibility of a real mass vaccination in endemic areas. The results obtained by research workers [11–16] give hints for development and testing such vaccines, above all concerning the possibility of direct stimulation of the immune mechanisms in the intestinal tract.

A WHO Scientific Group in 1969 recommended [7] that more potent vaccines should be developed and tested in small groups of volunteers to obtain basic information of the reactivity, acceptability, and immunogenic potency. The possible usefulness of several different vaccines such as adjuvant vaccines, toxin-toxoid vaccines and live attenuated vaccines should be investigated as well as live and killed oral vaccines.

According to the recommendations of the WHO and our own experience with production and control of Orovaccin®-E-Ruhr [17, 18] and Orovaccin®-Typhus [19], both produced by VEB Sächsisches Serumwerk, Dresden, a technology has been elaborated for the production of an inactivated whole cholera vaccine for oral administration [20]. The first batches were tested under laboratory conditions. The vaccine then showed a good compatibility

in volunteers without any side reaction. Similarly as in the preparations mentioned above, the antigen is incorporated into enteric coated tablets. The inactivated, dried and powdered antigen consists of equal parts of four *Vibrio cholerae* strains, the serotypes Inaba and Ogawa classical and biotype El Tor, respectively. One coated tablet contains 0.015 g antigen corresponding to 10×10^{10} to 12×10^{10} germs, calculated according to the International Opacity Standard (10 Opacity Units = 2.31×10^9 vibrios).

The results of the laboratory control were as follows. 1. The vaccine was not contaminated with viable vibrios. 2. Moisture content of the antigen was between 5.05 and 8.85%. 3. The LD₅₀ per 20 g albino mouse after intraperitoneal administration was: Antigen Inaba, classical 24.6 mg; Antigen Ogawa, classical 23.0 mg; Antigen Inaba, El Tor 30.3 mg; Antigen Ogawa, El Tor 24.6 mg; mixture of equal amounts of these antigens: 32.5 mg. 4. Orally administered antigen (1/2 coated tablet daily for 5 consecutive days) caused no side effects in mice. 5. Rabbit immune sera gave haemagglutinin titres against the four different strains between 1 : 80 and 1 : 640. 6. Thirty-five adult volunteers received 3 or 5 coated tablets on three consecutive days.

Blood sampling was made prior to immunization and 2 and 3 weeks afterwards. Agglutination, haemagglutination and vibriocidal titrations against Ogawa and Inaba antigens were performed with each serum sample.

All samples were tested under identical conditions. Ten of 14 serum pairs showed an antibody increase of at least two dilution steps by vibriocidal titration.

Further investigations are in progress to verify the specific humoral antibody response. Finally, controlled field trials with relevant epidemiological analyses will be necessary to evaluate the efficacy of the vaccine. It will not be possible to carry out such tests in Europe, as they require a great number of volunteers and a considerable period of observation. Only after a sufficient number of cholera cases have occurred will it be possible to judge reliably the epidemiological efficacy of the vaccine. This will be a difficult task even in endemic areas. We shall therefore have to perform special laboratory controls to prove the vaccine's sterility, toxicity and immunogenicity, and especially its ability of inducing an immune response in the intestinal lumen or in the tissues.

According to the results obtained in laboratory and field studies made with Orovaccin®-E-Ruhr and Orovaccin®-Typhus, it is believed that Orovaccin®-Cholera too has a protective efficacy if enough antigen is applied in appropriately set intervals.

It is recommended to vaccinate endangered persons with 3 coated tablets daily on three consecutive days and to give the same dose as booster every sixth month.

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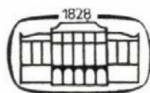
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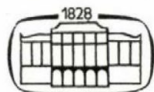
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TO THE SEVENTIETH ANNIVERSARY OF PROFESSOR GYÖRGY IVÁNOVICS

The papers in this issue are dedicated to Professor GYÖRGY IVÁNOVICS, Director of the Institute of Microbiology at University Medical School of Szeged, Member of the Hungarian Academy of Sciences, President of the Society of Hungarian Microbiologist, Chief Editor of *Acta Microbiologica Academiae Scientiarum Hungaricae*, Member of the Editorial Boards of several periodicals, Honorary Doctor in Laws of the University of Glasgow, Associate Member of the *Société Française de Microbiologie* and of the Society for General Microbiology, London, — on the occasion of his 70th anniversary.



Dear Professor Ivánovics,

In the name of all your pupils who regard you as their master I should like to wish you every happiness on the occasion of your 70th anniversary.

Please accept as a homage the following papers written by your former and present collaborators. We are proud of having the possibility to work in your close proximity and under your guidance inspired by your scientific enthusiasm. The pleasant atmosphere created by you has considerably contributed to the creative work of all your collaborators. In addition, we must thank you for always having had time to listen to the problems of your pupils and for promoting their progress.

Ad multos annos!

Ilona Béládi

June 11, 1974

[¹⁴C]ADENINE UPTAKE INTO BACILLUS ANTHRACIS ADENYLSUCCINATE LYASE DEFICIENT MUTANT

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(Received June 5, 1973)

Summary. The *Bacillus anthracis* adenylysuccinate lyase (EC 4.3.2.2.) deficient mutant utilized part of labelled adenine for growth and converted part of it to hypoxanthine. Multiplication of the mutant in the presence of ¹⁴C adenine ceased at a low level, although a considerable amount of free adenine was still demonstrable in the medium. The sonicated cell suspension of the mutant caused the conversion of labelled adenine to inosine and, subsequently, to hypoxanthine: traces of adenine nucleotides were also formed. The cell extract synthesized adenine nucleotides from adenine in the presence of exogenous ATP. Ribose-5-phosphate enhanced the nucleotide synthesis stimulating effect of ATP. The results suggest that adenosine deaminase activity on the one hand and the exhaustion of the 5-phosphoribosyl-pyrophosphate pool on the other may be the factors limiting the mutant's growth, viz. adenine incorporation.

IVÁNOVICS *et al.* prepared several auxotroph mutants from the non-capsulogenic Vollum strain of *Bacillus anthracis* [1]. The isolated adenine requiring mutants, although they produced capsule and toxin, were avirulent for animals [2, 3]. These mutants, in which the transformation of inosylic to adenylic acid is inhibited, could be divided into two groups, characterized by adenylysuccinate synthetase and lyase enzyme deficiency, respectively. Multiplication of spores administered into the abdominal cavity of mice was not followed by bacteraemia despite the simultaneous administration of a large amount of exogenous adenine [2]. Both groups of the adenine auxotroph mutants showed a partial de-repression of alkaline phosphatase synthesis [4]. The de-repressed alkaline phosphatase synthesis of the adenylysuccinate lyase deficient mutant could be repressed with adenine and adenine nucleotides [5]. Growth and ATP synthesis of the mutant *in vitro* were weaker than of those of the prototroph strain. The presence of adenosine deaminase and nucleoside phosphorylase may have reduced the synthesis of nucleotide from exogenous adenine by the mutant [6].

The aim of the present experiments was a study of the process of adenine uptake and of the roles of alkaline phosphatase, adenosine deaminase and nucleoside phosphorylase in the suboptimal growth of the mutant.

Materials and methods

Strain. The adenylysuccinate lyase deficient mutant of the non-capsulogenic Vollum strain of *B. anthracis* (23 C⁻ ade⁻), isolated [1] and characterized [2] by IVÁNOVICS *et al.* was used throughout.

Medium. Lactalbumin hydrolysate dephosphorylated with barium hydroxide [7] was completed with various salts, amino acids, glucose, vitamin B₁ and various quantities of adenine, according to the needs the individual of experiments [4]. Inorganic orthophosphate concentration was increased to 1.0 mM by the addition of 100 mM K₂HPO₄ solution.

Cultivation. The adenine concentration of the lactalbumin medium was increased from the original 10 µg/ml to 100 µg/ml. Ten ml amounts of the completed medium were distributed into 100 ml Erlenmeyer flasks. The content of each flask was inoculated with 10⁵ spores and the cultures were incubated in a reciprocal shaker under aeration in a water bath at 37°C for 16 hr. Optical density of the cultures was determined every hour, using a Magnephot II apparatus (Orion, Hungary).

[¹⁴C]adenine uptake and metabolism. The ¹⁴C adenine solution (Amersham) contained 500 µg/ml adenine (27.9 µCi/ml). The labelled adenine was diluted in cold adenine, if required. Optical density of the cultures growing in the presence of 10–100 µg/ml [¹⁴C] adenine was determined every hour and 0.5 ml samples were withdrawn at the same intervals for the determination of adenine uptake. Ten µl of the samples were passed through a millipore filter pad (HAWG, 0.2500 H. A. 0.45 µ) and washed in 1.0 ml cold saline. The filter pad was placed in a Packard cuvette, dried at 100°C for 20 min, then its activity was determined in 5.0 ml scintillation fluid. From the remaining samples bacteria were removed by filtration through a millipore filter and 10 µl amounts of the filtrates were chromatographed on a silica gel plate. The running solvent was so selected that adenine nucleotides would stay at the starting point, whereas adenine, adenosine, hypoxanthine and inosine migrated at different R_F values.

The silica gel GF₂₅₄ (Merck) thin-layers (100 × 200 × 0.25 mm) were activated for 30 min at 120°C. Five µg each of cold adenine, adenosine, hypoxanthine and inosine were applied at the starting points as tracers. Then 10 µl amounts of the labelled samples were applied to the starting points and run in n-butanol : ethyl acetate : methanol : ammonium hydroxide 25% (14 : 8 : 6 : 8) solvent [8]. The chromatograms were dried and the spots were marked in UV light. Appropriate reference standards were run with each series. The R_F-values obtained were, adenine = 0.68; adenosine = 0.57; hypoxanthine = 0.45; inosine = 0.23. The marked spots and start spots were scraped off and each was suspended in 5 ml scintillation fluid for one minute activity determination in a Packard Tri-Carb liquid scintillation spectrometer, Modell 2001. The scintillation fluid contained 0.5 g POPOP and 5.0 g PPO, dissolved in 1 litre toluene.

Preparation of crude bacterial extract. This was carried out by ultrasonic disintegration, as described previously [6]. The crude bacterial extracts were freshly prepared and their protein contents were determined before each experiment [9]. The extracts were utilized within 30 min after sonication.

Examination of [¹⁴C]adenine nucleotide synthesis. [¹⁴C]adenine nucleotide synthesis under the influence of sonicated cell suspensions of the adenine auxotroph mutant was examined under different conditions. [¹⁴C]adenine and, in certain experiments, adenosine and adenine nucleotides were added to the crude bacterial extract. In other experiments the combined effect of ATP and ribose-5-phosphate was studied. The systems were examined in 0.1 mM cacodylate buffer, pH 7.4 containing 10⁻³ M MgSO₄. Each sample was incubated in 500 µl volume at 37°C. Ten µl amounts of the samples withdrawn at appropriate intervals were chromatographed in silica gel thin-layer and the activities of the adenine nucleotides retained at the starting points were measured.

Quantitative determination of alkaline phosphatase in the bacterium extract. Enzyme activities of 200 or 250 µl cell extracts were determined. The amount of the extracts was made up to 2.0 ml with saline, then 2.0 ml of the substrate, containing 2.0% p-nitrophenylphosphate in 0.2 M Tris-HCl buffer pH 8.5, was added. The samples were incubated in a water bath of 37°C, for 30 or 60 min, depending of the schedule of the experiment. The enzyme reaction was stopped by the addition of 1.0 ml 1.0 N NaOH. Then the samples were centrifuged and the extinction of the supernatants was determined at 410 nm in a Spectromom 201 spectrophotometer. Enzyme activity was expressed in mµM p-nitrophenol released during incubation.

Results

In *B. anthracis* cultures grown in the presence of labelled adenine, the quantities of incorporated adenine and of its intermediary metabolites were estimated simultaneously with the measurement of optical density.

The adenine auxotroph mutant of *B. anthracis* was cultured in the presence of 10 $\mu\text{g/ml}$ (74 $\text{m}\mu\text{M/ml}$) labelled adenine. Adenine incorporation increased parallel with the increase of optical density. Growth reached its maximum in the 9th hour of incubation and by that time the cells had incorporated 72 $\text{m}\mu\text{M/ml}$ of adenine, practically the total amount (Fig. 1).

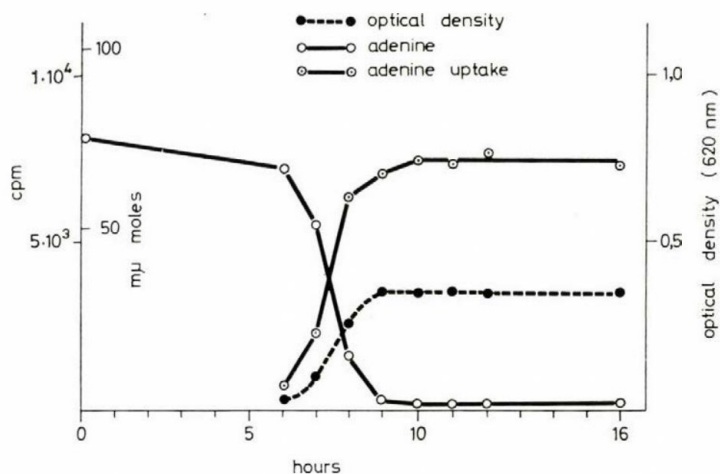


Fig. 1. Adenine uptake by developing culture of *B. anthracis* adenine auxotroph mutant in the presence of 10 $\mu\text{g/ml}$ [^{14}C]adenine. The cpm values are related to 10 μl , $\text{m}\mu\text{M}$ values to 1.0 ml of the sample

The optical density of the mutant culture grown in the presence of 100 $\mu\text{g/ml}$ (740 $\text{m}\mu\text{M/ml}$) adenine reached its maximum at 10 hr. No inosine was demonstrable in the logarithmic phase of growth. The quantity of incorporated adenine was 145 $\text{m}\mu\text{M/ml}$. A further decrease of the adenine concentration in the medium was followed by an increase of the hypoxanthine level. Although a considerable amount (395 $\text{m}\mu\text{M/ml}$) of unbound adenine was still present in the medium at 16 hr, neither bacterial growth nor adenine incorporation showed a further increase (Fig. 2). The experiment was also performed with a cell-free bacterial extract.

Mg^{++} was examined for its influence on ^{14}C adenine metabolism. The cell extract metabolized a considerable amount of adenine when Mg^{++} was present. The increase in the concentration of inosine and hypoxanthine was proportional to the decrease of the adenine level in both cases.

In the presence of EDTA, the cell extract scarcely influenced the metabolism of adenine.

The synthesized amounts of adenosine and adenine nucleotides could not be shown in the Figures owing to their very low activities. At 60 minutes of incubation, the concentration of adenosine was 0.5 $\text{m}\mu\text{M}$, that of adenine

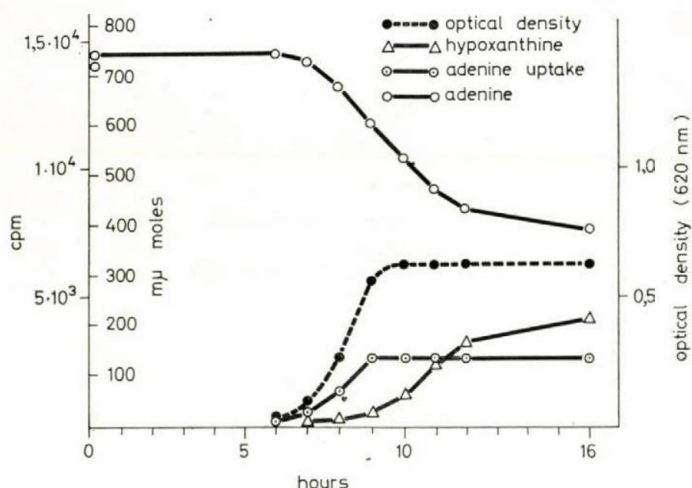


Fig. 2. Adenine uptake by developing culture of *B. anthracis* adenine auxotroph mutant in the presence of 100 $\mu\text{g/ml}$ [^{14}C]adenine. The cpm values are related to 10 μl , $\text{m}\mu\text{M}$ values to 1.0 ml of the sample

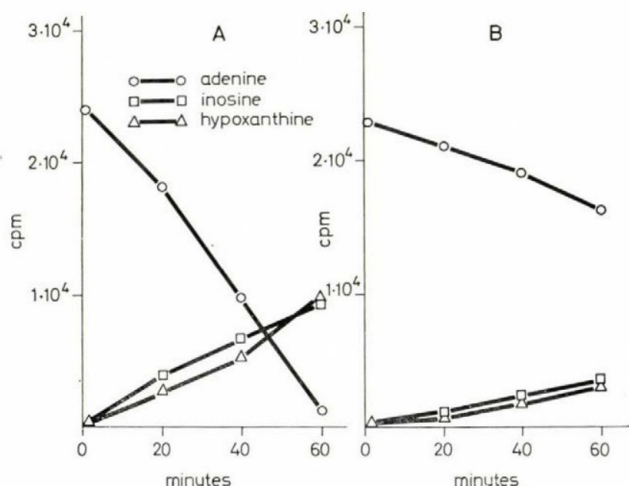


Fig. 3. Effect of *B. anthracis* adenine auxotroph cell extract on [^{14}C]adenine metabolism in the presence of Mg^{++} and EDTA; 250 μl cell extract, containing 4300 $\mu\text{g/ml}$ protein, were added to an equal volume of incubation mixture, which contained 110 $\text{m}\mu\text{M}$ [^{14}C]adenine (a), 500 $\text{m}\mu\text{M}$ MgSO_4 (b), and 500 $\text{m}\mu\text{M}$ EDTA dissolved in 250 μl 0.1 M, cacodylate buffer (pH 7.5); from each sample 10 μl aliquots were chromatographed. The cpm values are related to 10 μl

nucleotides 3.7 $\text{m}\mu\text{M}$ in the presence of Mg^{++} , whereas in the presence of EDTA, the respective quantities were 3.2 and 1.2 $\text{m}\mu\text{M}$ (Fig. 3).

In further experiments the effect of the mutant cell extract was studied on the synthesis of [^{14}C]adenine nucleotides. Exogenous adenosine and AMP

increased the metabolic rate of labelled adenine. With increased concentration of adenosine and AMP, the cell extract transformed a greater amount of labelled adenine to inosine and hypoxanthine. Activity of the 10 μ l sample was 3.4×10^4 cpm at 0-min. Under the applied conditions, the alkaline phosphatase

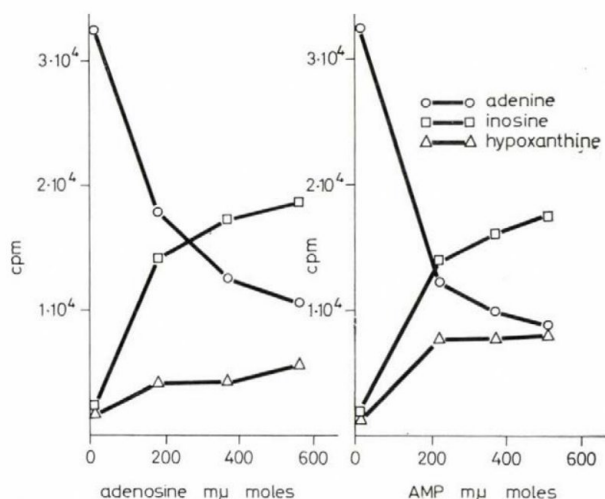


Fig. 4. Effect of *B. anthracis* adenine auxotroph cell extract on [¹⁴C]adenine metabolism in the presence of exogenous adenosine and AMP at various concentrations: 250 μ l cell extract, containing 4100 μ g/ml protein, were added to the incubation mixture, which contained 185 m μ M labelled adenine (a), 156–565 m μ M adenosine (b) and 215–509 m μ M AMP dissolved in 250 μ l 0.1 M cacodylate buffer (pH 7.5). The samples were incubated for 30 min and 10 μ l amounts were chromatographed. The cpm values are related to 10 μ l

present in 250 μ l bacterial extract decomposed 320 m μ M p-nitrophenylphosphate in 30 min (Fig. 4).

The cell extract of the *B. anthracis* adenine auxotroph mutant synthesized adenine nucleotides from labelled adenine in the presence of exogenous ATP; at the same time the increase of the ATP concentration also increased the metabolic rate of adenine. The results clearly showed that every enzyme playing a role in adenylic acid synthesis is present in the fresh bacterial extract. Exogenous adenosine, AMP and ATP are furnishing the energy necessary for the first energy requiring step. The rate of the process may be determined by the concentration of 5-phosphoribosyl pyrophosphate. Under the given conditions, the alkaline phosphatase present in 250 μ l bacterial extract decomposed 310 m μ M p-nitrophenyl phosphate in 30 min. The activity of 10 μ l sample was 2.3×10^4 cpm at 0-min (Fig. 5).

In further experiments, the pH optimum of ¹⁴C adenine nucleotide synthesis was examined under the influence of mutant cell extract, in the presence

of ATP. 0.1 M cacodylate buffer (pH range: 6.0–8.0) and 0.1 M Tris-HCl (pH 8.5) buffer were used in the experiments.

The maximum quantity of adenine nucleotides formed in the system was 6.0 μM . Alkaline phosphatase activity of the samples tended to rise in the pH-range from 6.0 to 8.5. The pH optimum for the transformation processes involved in adenine nucleotide synthesis ranged from 7.0 to 7.5.

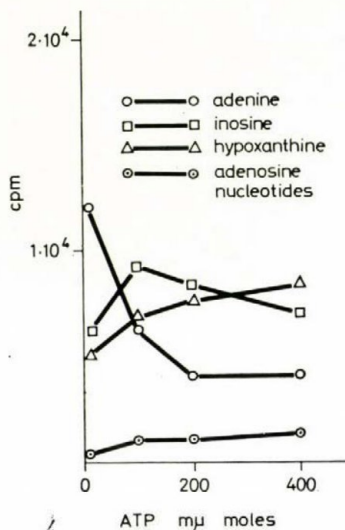


Fig. 5. Effect of *B. anthracis* adenine auxotroph cell extract on [^{14}C]adenine metabolism in the presence of exogenous ATP; 250 μl of the cell extract, containing 4100 $\mu\text{g}/\text{ml}$ protein, were added to the incubation mixture, which contained 110 μM labelled adenine and various quantities (range: 0–400 μM) of ATP dissolved in 250 μl 0.1 M cacodylate buffer, pH 7.5. The samples were incubated for 30 min and 10 μl amounts were chromatographed. The cpm values are related to 10 μl

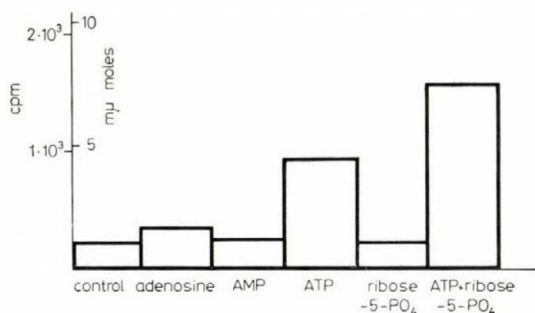


Fig. 6. Synthesis of [^{14}C]adenine nucleotides by *B. anthracis* adenine auxotroph cell extract in the presence of exogenous adenosine, AMP, ATP, ribose-5-phosphate and ATP + ribose-5-phosphate: 200 μl cell extract, containing 4200 $\mu\text{g}/\text{ml}$ protein, were added to each incubation mixture. These contained 110 μM labelled adenine and 200 μM adenosine, AMP, ATP, ribose-5-phosphate or ATP + ribose-5-phosphate, each dissolved in 300 μl 0.1 M cacodylate buffer, pH 7.5. The samples were incubated for 60 min and 10 μl amounts were chromatographed. The cpm values are related to 10 μl , the μM values to 0.5 ml of the sample

Equal concentrations of adenosine, AMP, ATP ribose-5-phosphate and ATP + ribose-5-phosphate were then tested each in itself for its effect on ^{14}C adenine nucleotide synthesis. Testing of the cell extract for alkaline phosphatase activity showed that 200 μl cell extract decomposed 585 $\text{m}\mu\text{M}$ p-nitrophenyl phosphate in 60 min (Fig. 6).

Ribose-5-phosphate, although ineffective in itself, was found to enhance the effect on ATP on nucleotide synthesis from labelled adenine. Adenosine and AMP had no influence on nucleotide synthesis. The fact that an equimolar concentration of ribose-5-phosphate acted as an adjuvant of exogenous ATP in increasing the mutant's adenine nucleotide synthesis, weighs in favour of the role of phosphoribosyl pyrophosphate PRPP in the incorporation of adenine.

Discussion

Loss of the bifunctional deacylating enzyme, adenylosuccinate lyase (E.C. 4.3.2.2.) brought about a double block in the *de novo* adenine nucleotide synthesis of *Escherichia coli* and *Salmonella typhi-murium* mutants, which thus required exogenous adenine for growth [10].

Uptake of adenine by the adenylosuccinate lyase deficient adenine auxotroph mutant of *B. anthracis* [2] was proportional to bacterial growth. In the early stage of culturing, adenosine, inosine and hypoxanthine were not demonstrable in the medium. These findings suggest that adenine incorporation takes place in a single step, probably with the aid of adenylophosphoribosyl transferase (E.C. 2.4.2.7.). This enzyme is known to catalyze the incorporation of adenine by various bacteria such as *E. coli* [11, 12], *S. typhi-murium* [13] and *B. subtilis* [14], and to produce adenylic acid from adenine and PRPP. PRPP is not only required for the utilization of exogenous purines, but it is an essential substance in the *de novo* synthesis of purine [15]. The exogenous purines, as end products, inhibit the synthesis of the early precursors, because the bulk of PRPP is utilized for purine incorporation, resulting in a quantitative decrease of *de novo* synthesized purines [16].

Synthesis of adenine nucleotides from ^{14}C adenine by the adenine auxotroph mutant of *B. anthracis* increased quantitatively in the presence of ATP + + ribose-5-phosphate. The underlying mechanism was probably the formation of PRPP from ATP and ribose-5-phosphate under the influence of the cell extract.

Phosphoribosyl pyrophosphate synthetase (E.C. 2.7.6.1.), which synthesizes PRPP from ATP and ribose-5-phosphate, was first demonstrated by KORNBERG *et al.* in the pigeon liver [17]. Recently, this enzyme has been prepared from various bacteria such as *S. typhi-murium* [18] and *E. coli* [19].

The growth requirements of the *B. anthracis* adenine auxotroph strain could also be satisfied with adenosine [2], although to a lesser degree than by

adenine itself; this may be attributed to adenosine deaminase production by the strain, or to a weaker penetration capacity of adenosine [6]. *E. coli* strain K₁₂, not containing adenosine deaminase, was found to incorporate adenosine in two steps. First, adenosine is converted to adenine by adenosine phosphorylase, and then the transformation of adenine to AMP is catalyzed by adenine phosphoribosyl transferase [20, 21].

B. cereus, which is phylogenetically closely related to *B. anthracis*, was found to contain, apart from adenosine deaminase, also adenosine ribosidase, an enzyme which splits adenosine into adenine and ribose [22].

In yeast cells and animal tissues, exogenous ATP was shown to allow a direct transformation of adenosine to AMP in the presence of adenosine kinase [23].

The *de novo* adenine nucleotide synthesis of the *B. anthracis* adenine auxotroph strain is blocked by mutation. The capacity of the remaining biosynthetic pathway ensures a lower nucleotide pool than that available in the prototroph strain. This low adenine nucleotide pool then results in a de-repression of alkaline phosphatase synthesis [5].

The adenylic acid synthesized by the mutant may transform into adenosine under the influence of alkaline phosphatase. The substrate specificity of *B. anthracis* alkaline phosphatase is almost identical for adenylic acid and p-nitrophenyl phosphate [24]. Adenosine is converted to inosine by the cellular adenosine deaminase and the inosine thus formed is converted to hypoxanthine by nucleoside phosphorylase.

Inosine and hypoxanthine cannot be utilized by the mutant for nucleotide synthesis or growth. Exhaustion of the PRPP pool seems to be the factor limiting nucleotide synthesis or adenine incorporation by the mutant. This hypothesis agrees well with the experimental observation that neither adenine incorporation, nor the growth of the auxotroph strain, cultured in the presence of a considerable amount of labelled adenine, reached the growth of the prototroph, although much free adenine was still present in the medium after 16 hr incubation. Alkaline phosphatase synthesis became de-repressed in the *B. anthracis* adenine auxotroph mutant [4]. The affected metabolism of the mutant brought about morphologically demonstrable changes, which manifested themselves with a loosening and injury of the cell wall structure [7], and a gradual disintegration of the bacterial cells [2], arising probably as a consequence of de-repressed alkaline phosphatase synthesis. These changes are the result of a mutation of a single structural gene, the adenylysuccinate lyase gene, which finally leads to a complete loss of the virulence of the bacterial strain.

Acknowledgement. We are indebted to Professor G. IVÁNOVICS for valuable advices.

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STUDY OF LYSOGENIC *BACILLUS CEREUS* STRAINS BY ELECTRON MICROSCOPY

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(Received October 5, 1973)

Summary. Mitomycin C caused rapid lysis in exponential phase cultures of 10 different *Bacillus cereus* strains. While examining the lysates of these strains we have attempted to detect phages on *B. cereus*, *B. subtilis*, *B. anthracis* and *B. megaterium* as hosts. In 3 strains was it only possible to demonstrate phages released during lysis. *B. cereus* strains 4, 5P₃ and 5P₁₅ released two, morphologically different, kinds of phage particles. "Cured" cultures not inducible with mitomycin C were isolated by the use of acridine orange from 5 lysogenic strains. Two of these were sensitive to some phages obtained by induction from lysogenic strains.

Lysogeny is not infrequent among *Bacillus cereus* strains [1—4]. Most authors were able to demonstrate the presence of phages acting on suitable indicator cultures. ALTENBERN [4] observed induction by UV irradiation of several *B. cereus* strains but failed to demonstrate phages in lack of sensitive indicator strains. In our 10 *B. cereus* strains, mitomycin C caused rapid lysis after a short residual growth. In 7 of the 10 cultures phages were not demonstrable on about 50 *Bacillaceae* strains used as indicator. The present paper deals with electron microscopic studies of concentrated and purified lysates of these *B. cereus* strains and with the characteristic features of the isolated, "cured" derivatives of the lysogenic strains.

Materials and methods

Bacterial strains. *B. cereus* strains 569, 5P₃ and 5P₁₅ were obtained from the collection of Professor M. POLLOCK, Edinburgh. Seven strains (*B. cereus* 4, 7, 8, CA 10, CA 82, 114a and 116) were isolated in this institute [5]. As indicator cultures, various *B. cereus*, *B. anthracis*, *B. megaterium*, *B. subtilis* and *B. licheniformis* strains of our collection were used.

Culture media. Yeast extract peptone broth [6] and solid medium prepared with 1.5% agar were used. Potato medium was made by boiling 200 g potatoes in 1 litre tap water for 50 min, adding 5 mg MgSO₄ per litre and adjusting the pH to 6.8.

Cultivation and induction of bacteria were carried out as described in a previous paper [7], with the exception that in order to obtain a high quantity of lysates, 200 ml YP broth cultures were shaken in 1 litre Erlenmeyer flasks in water bath at 37°C. Optical density was determined by a Bausch and Lomb Spectronic 20 photometer at 620 nm. As an inducing agent mitomycin C (Kyoma) was added at 0.2 OD in a final concentration of 0.5 µg/ml. Growth and lysis of the cultures were followed by recording their optical density.

Concentration and purification of lysates for electron microscopy were carried out as described previously [8], but the lysates were concentrated to 1/200–1/400 of their original volume. After centrifugation in a Janetzki Vac 60 centrifuge at 30,000 g for 2 hr then at 8000 g for 10 min the concentrated lysate was dropped on a 400 mesh LKB grid covered with Formvar film strengthened by carbon. After 1 min adsorption the preparations were rinsed with

distilled water then stained negatively with 1% uranyl acetate [9] For electron microscopy, a JEM 100-B apparatus was used.

Attempts at detecting infective phage particles. After maximum lysis induced by mitomycin C, the lysates were passed through a membrane filter (Göttingen No. 6) and 10-fold serial dilutions were dropped on YP plates overlaid with soft agar containing the indicator cultures [10]. Phage titre of the lysates was determined by the plaque method using 10-fold serial dilutions and a sensitive indicator strain [10].

Isolation of prophage-free derivatives. Lysogenic *B. cereus* strains were cultured in the presence of acridine orange (10 µg/ml) in potato medium shaken at 37°C until sporulation. The cultures were then streaked on YP agar plates. Isolated colonies were inoculated into 5 ml YP broth and after shaking at 37°C, 0.5 µg/ml of mitomycin C was added to the young cultures in the exponential phase. Clones not lysed after mitomycin C treatment were regarded as "cured" derivatives of the lysogenic parent strains.

Results

Testing of lysates on indicator strains. Results obtained in this study were in agreement with previous findings [7] in that *B. cereus* strains proved inducible by mitomycin C. During our attempt at showing phages in the lysate it was confirmed that cultures 5P₃ and 5P₁₅ produced plaques on *B. anthracis*

Table I

Dimensions of B. cereus phages

Phage derived from		Head diameter, nm	Tail breadth, nm	Tail length, nm
4	α	73	13	200
	β	53	13	160
7		46	13	146
8		60	10	180
CA 10		54	6	120
CA 82		53	6	130
114 a		53	6	113
116		54	6	130
569		73	10	160
5P ₃	α	40/86	10	130
	β	46	6	186
5P ₁₅	α	33/66	6	120
	β	46	6	186

Davis at a titre of 10^2 – 10^4 /ml. Cross-tests were made between the lysates and the ten original *B. cereus* strains as indicators. In this way only one more strain (569) was found to produce plaques on *B. cereus* 4 strain as indicator. This phage could be propagated by the single plaque method; the phage material obtained in this manner contained 10^6 p.f.u./ml. The lysates of the other 7

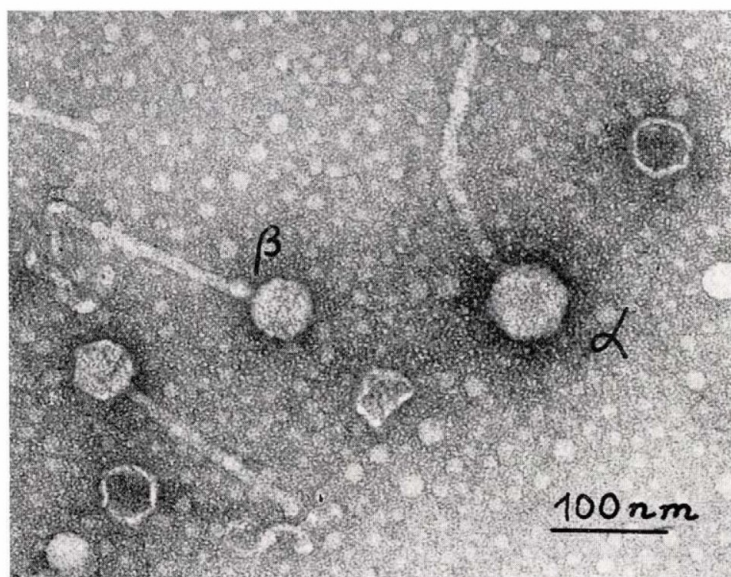


Fig. 1. Electron micrograph of the concentrated lysate of *B. cereus* 4 ($\times 150\,000$)

strains failed to show phage activity on 14 *B. cereus*, 17 *B. anthracis*, 5 *B. subtilis*, 5 *B. licheniformis* and 7 *B. megaterium* strains. Even in the case of the *B. cereus* strains 5P₃, 5P₁₅ and 569 it was impossible to find further indicator strains except those mentioned above.

Electron microscopy. All lysates obtained by mitomycin C induction contained intact phage particles. In the lysate of *B. cereus* 4, two types of phage different in size were present. A considerably larger particle (α) occurred in lower numbers, while the smaller particle (β) was more frequent; it was characterized by an undulating filament at the end of the tail (Fig. 1). Phages isolated from *B. cereus* 7 and 8 showed similar filaments (Fig. 2). Concerning size of their heads and tails, these three phages with undulating filaments were different (Table I).

Phages obtained from the lysates of *B. cereus* strains CA 10, CA 82, 114a and 116 were practically identical in morphology and size (Fig. 3, Table I). They were characterized by a regular hexagonal head and a thin, usually

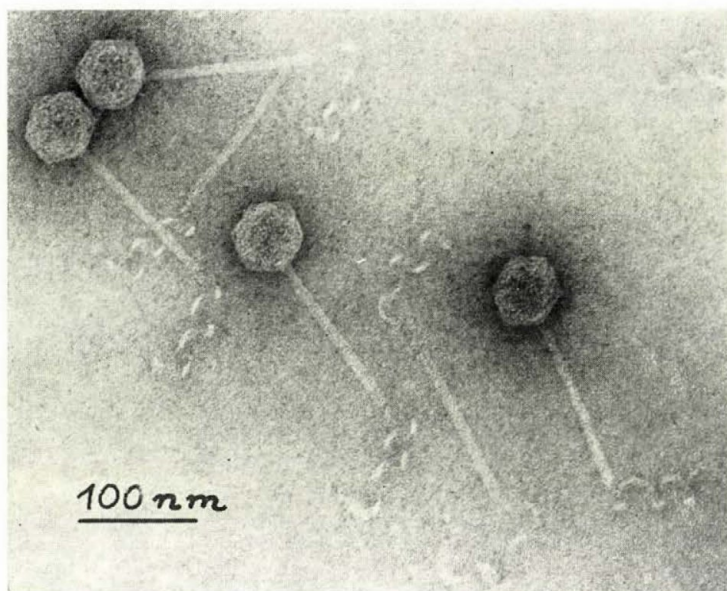


Fig. 2. Electron micrograph of the concentrated lysate of *B. cereus* 8 ($\times 150\,000$)

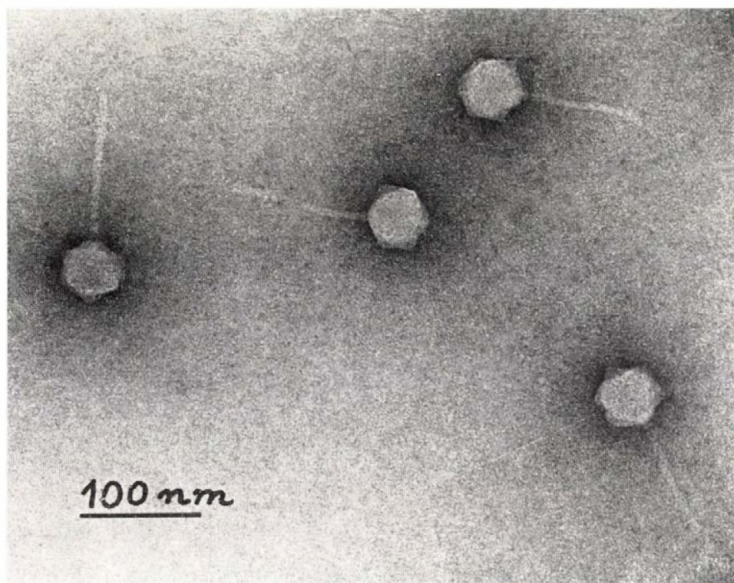


Fig. 3. Electron micrograph of the concentrated lysate of *B. cereus* CA 82 ($\times 150\,000$)

cross-striated, tail. *B. cereus* 569 produced a morphologically similar but larger phage (Table I).

Lysates of *B. cereus* 5P₃ and 5P₁₅ contained two different phage particles (Fig. 4). In both lysates, particles with an elongated head (α) were predominat-

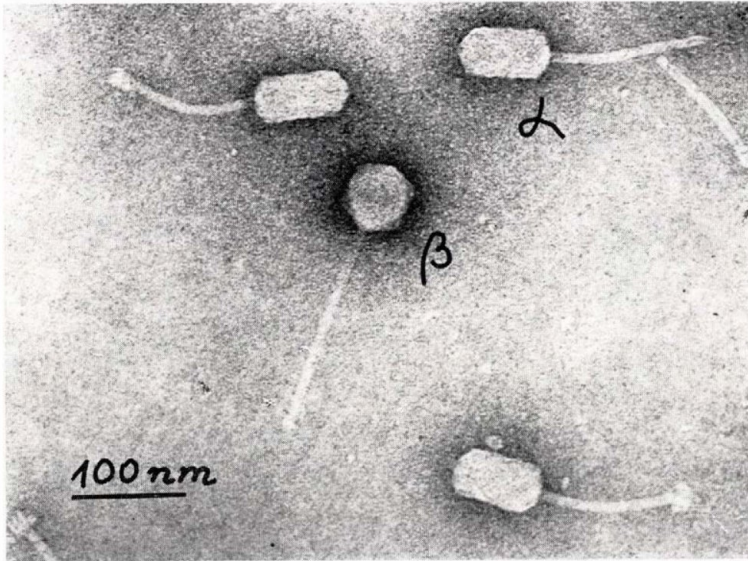


Fig. 4. Electron micrograph of the concentrated lysate of *B. cereus* 5P₃ ($\times 150\,000$)

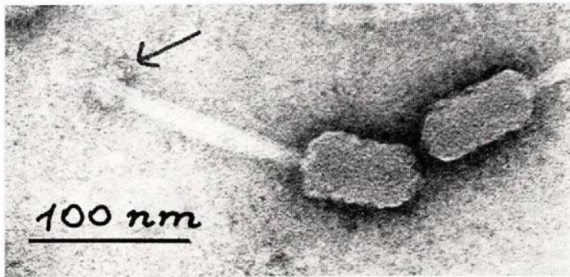


Fig. 5. Electron micrograph of α phage isolated from *B. cereus* 5P₁₅ ($\times 210\,000$)

ing but the phage of 5P₃ was larger than that of 5P₁₅. At the end of the tail of these phages a definite knob was visible (Fig. 5). The other type of phage produced by these strains (β) had a regular hexagonal head and a fairly long tail.

The values shown in Table I represent the average of 25–30 measurements. In view of their hexagonal head and non-contractile tail, all the isolated phages belonged to BRADLEY's group B [11].

Attempts at isolating phage-free derivatives. To obtain "cured" cultures not inducible with mitomycin C, 200 colonies of each of the 7 strains were examined. Each of the strains *B. cereus* CA 10, CA 82, 114a and 116 yielded one, while strain *B. cereus* 8 two, cured derivatives. Fig. 6 shows the growth

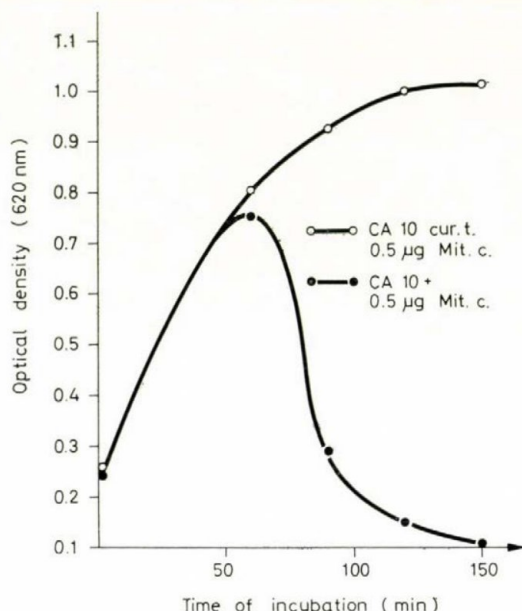


Fig. 6. Growth curves of *B. cereus* strains CA 10 and CA 10 *cur*; mitomycin C (0.5 µg/ml) was added at 0 min

curves of the parent and cured (*cur*) culture of strain CA 10 after the addition of 0.5 µg/ml of mitomycin C. After 150 min incubation the parent strain showed an almost complete lysis while the "cured" culture was unaffected. A similar behaviour was recorded in the case of all the other "cured" strains even after several subcultures. Attempts to isolate "cured" cultures from *B. cereus* strains 4 and 7 have failed.

The effect of the lysates of the parent *B. cereus* strain on the "cured" cultures is shown in Table II. Four of the lysates acted upon the "cured" derivatives of CA 10 and 116: up to 10^4 dilution the lysates produced isolated plaques; at lower dilutions confluent lysis was seen. No phage action was demonstrable on two "cured" derivatives of strain 8 and on 114a *cur*. The question marks in Table II mean that the corresponding undiluted lysate produced a faint smooth area seemingly less dense on the soft agar layer inoculated with the indicator strain. This reaction cannot be regarded as phage action, as the culture started from this site failed to show lysis after mitomycin C treatment

Table II

Effect of *B. cereus* strain lysates on "cured" derivatives of *B. cereus* strains and on *B. cereus* 4 and *B. anthracis* Davis

Lysate	Indicator strain						
	<i>B. cereus</i>						<i>B. anthracis</i>
	8 cur No. 7	8 cur No. 8	CA 10 cur	114 a cur	116 cur	4	Davis
4	—	—	—	?	—	—	—
7	?	?	—	—	—	—	—
8	—	—	—	?	—	—	—
CA 10	—	—	+	—	+	—	—
CA 82	—	—	+	—	+	—	—
114 a	—	—	+	—	+	—	—
116	—	—	+	—	+	—	—
569	—	—	—	—	—	+	—
5P ₃	—	—	—	—	—	—	+
5P ₁₅	—	—	—	—	—	—	—

+ Phage activity at 10^{-4} dilution of the lysate.

— No phage activity.

? Less dense smooth area with undiluted lysate.

and the supernatant had no effect whatever on the corresponding indicator strain. The reaction was probably due to an unidentified bacteriocin effect.

Discussion

All *B. cereus* strains examined in this study were inducible with mitomycin C. In this respect these strains resembled the *B. cereus* cultures of ALTENBERN [4] and ALTENBERN and STULL [12] who used UV irradiation as an inducing agent. In their experiments it was only on one occasion that they found an indicator strain for one supposed phage.

Electron microscopy revealed that our *B. cereus* strains released complete phage particles belonging to BRADLEY's group B. The undulating filament demonstrated in 3 different phages was probably analogous to the similar structures shown in *Serratia marcescens* phage SM2 which, according to BRADLEY, are "unwinding strings of tail capsomers rather than functional tail fibres as with the T even phages" [13]. Phages of strains 5P₃ and 5P₁₅ characterized by elongated heads and definite knobs at the end of the tails resemble BRADLEY's staphylococcal and pseudomonad phages [14].

Phages released by mitomycin C induction from *B. cereus* are highly specific. Examining the phages released from these 10 *B. cereus* strains, corresponding indicator strains were found only for 3 of them.

WALZ and SAZ [15] isolated from the asporogenic mutant of *B. cereus* 569 a phage that contained 5-hydroxy-methyluracil in its DNA instead of thymine. This phage showed a close immunobiological and DNA-constitutional relationship to *B. subtilis* phage SP 32. As WALZ and SAZ have shown that their phage propagates on a number of *B. cereus*, *B. licheniformis* and *B. subtilis* strains, it does not seem identical with the phage isolated by us which was inactive against all indicator strains except *B. cereus* 4.

The high specificity of prophages carried by *B. cereus* strains was remarkable. Undoubtedly, some prophages yield immunity against temperate vegetative phages of the homologous and of some heterologous strains. Phage resistance cannot, however, always be explained by prophage immunity: namely 3 of the 5 "cured" strains that were not inducible with mitomycin C and had, therefore, probably been freed from their prophage, remained resistant to the homologous and to certain heterologous phages.

It is known that phages acting on *B. cereus* are common in nature and easy to isolate from soil samples. At the same time, suitable indicator strains for vegetative forms derived from prophages of *B. cereus* strains lysogenized in nature, occur very unfrequently.

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KINETICS OF STEROID-DELTA-1-DEHYDROGENASE INDUCTION

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Summary. Inducible steroid-delta-1-dehydrogenase synthesis has been studied in continuous and batch cultures of *Arthrobacter simplex*. The inducer was added to batch cultures in the declining growth phase, while in the continuous cultures growth and induction proceeded simultaneously, as a one-stage continuous process. In both cases a positive correlation was found between the rates of growth and enzyme production. The correlation could be expressed in terms of the LUEDEKING—PIRET equation, and the mathematical characterization of the process permitted the conclusion that enzyme production is most intensive in rapidly growing cells, but it also takes place in non-growing cells. The mathematical treatment offered an explanation for the enhancement of enzyme production by methanol, which is a growth inhibitor.

The synthesis of steroid-delta-1-dehydrogenase is inducible in *Arthrobacter simplex*, like in many other bacteria [1]. When the bacterium is grown in the presence of the inducer, the enzyme can be regarded as the product of a fermentation process. To analyze the kinetics of product formation, the first requirement is to clarify whether the product is characteristically synthesized by growing or by non-growing cells or its formation equally associates with the phases of growth and non-growth [2]. The LUEDEKING—PIRET equation has been proposed as a kinetic model expressing the relationship between growth and production [3].

Studies of steroid-delta-1-dehydrogenase synthesis showed that the rate of enzyme production follows the growth rate, thus growth and production are in a positive correlation [4]. This seemed to contradict the experimental observation that when the inducer steroid is dissolved in methanol, the solvent enhances enzyme production, although it inhibits bacterial growth [5].

The present experiments were carried out to clarify the nature of the correlation between growth and enzyme production and to find an explanation for the stimulatory effect observed in the presence of methanol.

Materials and methods

Bacterial strain and culturing. *A. simplex* was grown at 30°C, under aeration, on a medium containing 1.0% casein hydrolysate (enzymic), 0.1% yeast extract, 0.5% sodium chloride, 0.025% magnesium sulphate, and 0.007% calcium chloride.

Inducer. For this purpose cortisol was used in aqueous suspension, which was added to the cultures so as to give a final concentration of 200 $\mu\text{g/ml}$.

Assessment of the bacterial mass. Extinction of appropriately diluted samples was read at 530 nm, multiplied by the degree of dilution and the cell mass was expressed in terms of optical density units.

Determination of enzyme content. The working principle was the same as proposed by KONDO [6]: 0.25 ml of the appropriately diluted culture was added to 4.25 ml phosphate buffer (0.03 M, pH 7.0), containing 10 $\mu\text{g/ml}$ 2,6-dichlorophenol-indophenol (DPIP); 0.2 mg cortisol in 0.2 ml methanol was then added to the mixture and 0.2 ml methanol in itself to the blank. The discoloration of DPIP was read at 600 nm for 10 min and the enzyme content was expressed as mU of activity required to dehydrogenate 0.001 μM cortisol, viz. to reduce an equivalent amount of DPIP, in one minute.

Assay of cortisol. Cortisol was dissolved in ethanol at an approximate concentration of 10 $\mu\text{g/ml}$ and the light absorption of the solution was read at 242 nm. The specific extinction is $E_{1\text{cm}}^{1\%} = 443$.

Assay of prednisolone. This was carried out by the method of MIZSEI and SZABÓ [7]. The bacterial suspension was extracted with ethyl acetate and an aliquot part of the extract was dried by evaporation. To the dry residue, 8 ml sulphuric acid-ethanol reagent was added, the mixture was incubated in a boiling water bath for 8 min, after which the colour intensity was read at 540 nm against a similarly prepared blank solution.

Results

To facilitate kinetic calculations, the LUEDEKING—PIRET equation was rearranged as follows.

$$\dot{p} = b_1 x + b_2 \dot{x}$$

$$\frac{\dot{p}}{x} = b_2 \frac{\dot{x}}{x} + b_1$$

where p is the enzyme concentration (mU ml^{-1}), and x the bacterium density (OD); $\frac{\dot{x}}{x}$ is further on designated as μ .

In the case of a continuous fermentation, the specific bacterial growth rate (μ) corresponds numerically with the dilution rate (D), and the specific production rate $\left(\frac{\dot{p}}{x}\right)$ can be calculated by multiplying the quotient of steady-state activity and steady-state bacterial density by the dilution rate $\left(D \frac{p}{x}\right)$.

The process of enzyme synthesis was first examined in batch cultures. The inducer was added after the critical x value, designated by KONO and ASAI as c_{xc} , had been reached. This occurred in the 17th hr of cultivation. Thus, induction was made in the declining growth phase, during which μ is a variable, whereby it becomes possible to check the fit of the equation. Induction lasted for 140 min and enzyme concentration was determined in samples taken at 20 min intervals. The time course of the enzyme level and the growth during induction are shown in Fig. 1a. Fig. 1b shows the specific growth rate and specific enzyme production rate — determined from the tangents — in the

function of time (Fig. 1). The fit of the equation was checked as shown in Fig. 2, where the values of $\frac{\dot{p}}{x}$ are plotted against μ (Fig. 2).

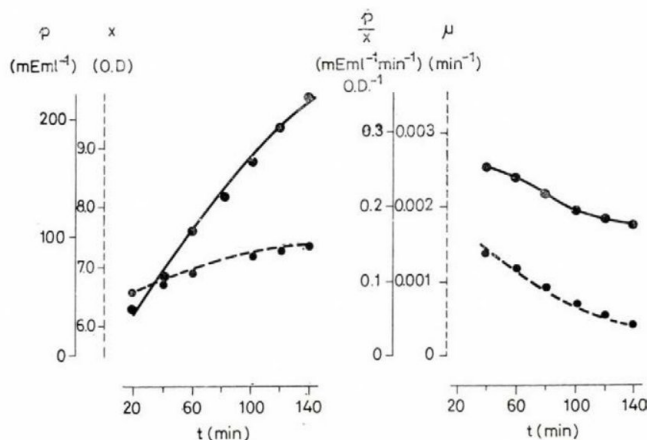


Fig. 1. Induction of steroid-delta-1-dehydrogenase from 17.20 to 19.20 hr of batch fermentation. (a) Time course of enzyme formation: ●—● and growth: ●-----● (b) Specific rate of enzyme production ●—● and specific rate of growth ●-----● against time

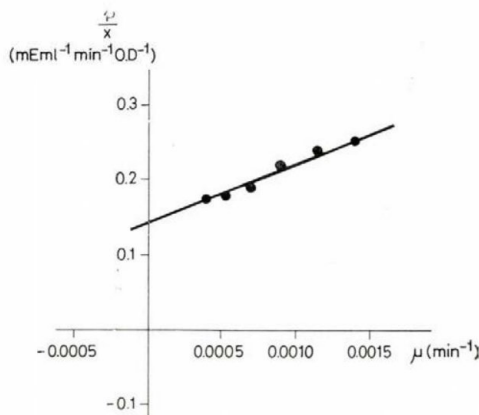


Fig. 2. Induction of steroid-delta-1-dehydrogenase from 17.20 to 19.20 hr of batch fermentation. Specific rate of enzyme production as a function of the specific growth rate. Parameters: $b_1 = 0.14$; $b_2 = 80.10$

In continuous fermentation systems, enzyme concentration and bacterial density were determined at three different dilution rates. Both cultivation and induction proceeded simultaneously as a one-stage process. Steady-state data are summarized in Table I, in which each x and p value represents the mean of 7 measurements (Table I).

Table I

Induction of steroid-delta-1-dehydrogenase in a continuous fermentation system

D(hr ⁻¹)	D(min ⁻¹)	x(OD)	p(mU ml ⁻¹)	$\frac{\dot{p}}{x}$ (mU ml ⁻¹ min ⁻¹ OD ⁻¹)
0.103	0.0017	7.09	360.0	0.086
0.072	0.0012	7.45	412.2	0.067
0.052	0.0008	8.12	501.0	0.049

Characteristics of the strain and medium used: $\mu_{\max} = 0.0038 \text{ min}^{-1}$; $c_{xc} = 6.70$.
 Calculated parameters: $b_1 = 0.019$; $b_2 = 39.32$

The effect of methanol was studied under the same conditions of batch fermentation as above, using 3.0 or 5.0% methanol. The $\frac{\dot{p}}{x}$ values plotted against μ can be seen from Fig. 3; it seems that the enzyme production enhancing effect of methanol is associated with the b_1 value.

In the last experiment, the methanol effect responsible for the increase of the b_1 value was studied in greater detail. The rate of the measurable processes of induction, in which the inducer is taking part, was therefore determined. Bacterial cell suspensions were prepared in an aqueous solution of cortisol (300 $\mu\text{g/ml}$) and appropriate amounts of methanol (range: 0.0–6.0%) were added to each system. The suspensions were incubated under aeration at 30°C for 140 minutes. During this time no measurable bacterial growth took place. The following processes were studied: (1) specific enzyme production

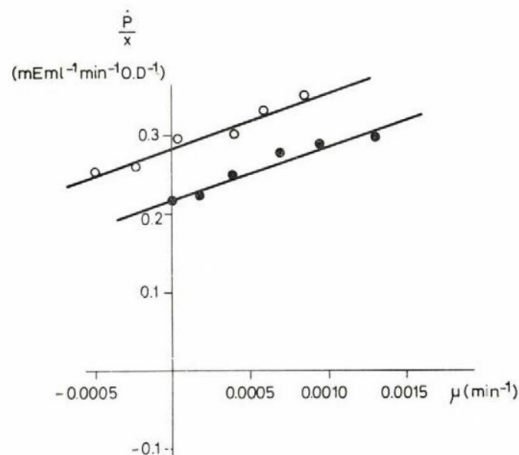


Fig. 3. Induction of steroid-delta-1-dehydrogenase in the presence of methanol, from 17.20 to 19.20 hr of batch fermentation. Specific rate of enzyme production as a function of the growth rate, in the presence of 3.0% ●—● and 5.0% ○—○ methanol. Parameters at 3.0% methanol: $b_1 = 0.22$; $b_2 = 66$ –31; at 5.0% methanol: $b_1 = 0.28$; $b_2 = 71.55$

rate; (2) specific rate of cortisol dehydrogenation; *viz.* prednisolone production; and (3) the specific rate of cortisol decomposition. The rate of dehydrogenation was studied also after preliminary induction of bacteria suspended in chloramphenicol solution (100 µg/ml). In this particular case no induction took place during dehydrogenation. The data are summarized in Table II, which also shows the cortisol saturation levels corresponding to the different concentrations of methanol used. Each assay was performed in two to four replicas.

Table II

Effect of methanol on the processes taking place in the course of steroid-delta-1-dehydrogenase induction

Methanol, per cent	0.0	1.0	2.0	3.0	4.0	5.0	6.0
Water-solubility of cortisol µg ml ⁻¹	370 320	400 335	420 350	480 365	— 380	520 410	— 415
Enzyme formation mU ml ⁻¹ min ⁻¹ OD ⁻¹	0.08 0.08 0.11 0.07	0.09 0.10 0.13 0.08	0.11 — 0.17 0.09	0.13 0.11 0.21 0.10	— — — 0.12	0.17 0.13 0.24 0.13	— — 0.26 0.13
Decomposition of inducer µgml ⁻¹ min ⁻¹ OD ⁻¹	0.11 —	— 0.14	0.11 0.16	0.13 —	0.15 —	0.16 —	0.16 0.21
Dehydrogenation of inducer µg ml ⁻¹ min ⁻¹ OD ⁻¹	3.87	—	4.18	—	4.76	—	5.20
Dehydrogenation (in presende of chloramphenicol) µg ml ⁻¹ min ⁻¹ OD ⁻¹	15.6 8.7 13.8	— — —	14.4 — 13.7	14.4 8.0 —	14.4 — 13.8	14.2 7.5 —	13.3 — 13.8

Discussion

The correlation between inducible enzyme synthesis and growth has been studied mainly under conditions of continuous culturing. Activity changes in the function of the dilution rate (*D*), *viz.* the specific growth rate, were reported for several enzymes [9–12].

The enzymes studied were catabolic enzymes, the formation of which are inhibited by catabolic repression at high dilution rates [13]. Apart from the inhibited ranges, a positive correlation seems to exist in most cases between the rates of growth and enzyme production. This is obvious in cases when increasing *D* caused either no decrease, or even an increase of enzyme activity; in our opinion, even the fall of the enzyme level does not exclude a positive correlation between the above rates.

The inducible synthesis of steroid-delta-1-dehydrogenase offers a suitable model for correlation studies, because the inducer is not an essential nu-

trient, the enzyme formed does not influence growth and no catabolic repression takes place if an appropriate medium is used. In the present studies, the inducer was added in excess to batch cultures and in repeated doses to one-stage continuous cultures. Owing to decomposition by the bacteria, the concentration of the inducer fluctuated around 20 $\mu\text{g/ml}$ in the continuous fermentation experiment.

The results disclosed a linear relationship between specific growth rate and specific enzyme production rate in both continuous and batch culture experiments. The equation describing this relationship corresponds to the LUEDEKING—PIRET equation; it expresses the proportional increase or decrease of the enzyme production rate with cell count and cell growth and clearly shows that growing and non-growing cells are both capable of enzyme synthesis (at $\dot{x} = 0$, $\dot{p} > 0$, if $b_1 > 0$). This agrees well with the reported observations.

If specific activity is plotted against D , the induction of steroid- δ -1-dehydrogenase produces an initially steeply descending curve which at low D values becomes gradual. The curve resembles the one for beta-galactosidase when induced in a nitrogen-limited chemostat [13]. The process characterized by the LUEDEKING—PIRET equation is just an example of such growth-correlated inductions where an increase in D leads to a decrease in activity. The initial rapid decrease is due to enzyme production by the non-growing cells.

The mathematical characterization of a process permits further conclusions to its details. This was utilized in the present study for obtaining more information about the role of methanol.

Methanol was found to increase the enzyme production of *A. simplex*, although it inhibited its growth. Mathematically, this is expressed by the decrease of the term for growth, while the values of the parameter b_1 increases markedly. The specific rate of enzyme production by cells in the resting phase (e.g. in water-suspended cells) is equal to $b_1 \left(\text{if } b_{2\mu} = 0, \frac{\dot{p}}{x} = b_1 \right)$. Methanol enhanced the enzyme synthesis also in water-suspended cells, thus the increase of b_1 was unequivocal in this case as well.

The purpose of the last experiment was to find a biological explanation of the b_1 -increasing effect. The value of b_1 was considerably influenced by the concentration of the inducer (see batch induction: $b_1 = 0.14$, continuous induction: $b_1 = 0.019$). It was therefore postulated that methanol promotes the uptake of inducer by the cells.

There is experimental evidence of the water-solubility of cortisol being increased by methanol. On its addition to an aqueous solution of cortisol, methanol causes a proportional increase in the rate of induction and of cortisol decomposition as well. Both intracellular processes are equally enhanced. The dehydrogenase-containing cells oxidize the dissolved cortisol independ-

ently of the concentration of methanol present. The rate of this process is by two orders higher than those of the intracellular processes. Thus the question may justly arise whether the developed enzyme still requires the intake of substrate.

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A THEORETICAL TREATMENT OF THE PROBLEM OF VIRION ATTACHMENT AND INFECTION OF THE HOST CELL

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Summary. Analysis of mathematical treatments proposed for the description of diffusion limited reactions at the surface of small spherical bodies has shown that in contrast to several previous attempts these cannot be directly applied for the characterization of initial animal virion–host cell interactions. Experimental results obtained with polio virion–host cell systems treated with certain local or general membrane stabilizers have allowed an individual visualization of the roles of some of the microscopic parameters controlling a successful, complementary interaction between virion and host cell. A theory is advanced, based on the interpretation of receptors of an animal cell as metastable, transient conformational states.

The classical model for the description of initial virion–host cell interaction has been based chiefly on studies of phage–bacterium systems. The following essential points emerged from these studies.

1. The equation describing the change of the free initial virion concentration per cell V_i in function of time t is formally identical with the equation of the pseudo-first-order irreversible chemical reaction

$$\frac{V_t}{V_i} = e^{-kt} \quad (1)$$

where k is the apparent reaction rate constant and V_t the free virion concentration per cell at t time.

2. Practically any cell with at least one active virion attached (expressed as p.f.u.) is *de facto* infected and represents one infectious centre.

3. The frequency of infectious centres per cell IC for any average number of virions attached per cell $\bar{V}_a$ follows the Poissonian distribution and can be calculated with the equation

$$p_z = 1 - e^{-\bar{V}_a} \quad (2)$$

where z means that at least one virion is attached per cell, thus

$$p_z = IC \text{ or } p_z \sim IC \quad (3)$$

In view of its importance for our further considerations, let us look somewhat closer at the meaning of the apparent rate constant k in equation (1).

In virology, the "reaction rate" is usually determined in systems where the cellular receptors (defined as cell count) are present in excess (each cell carries several hundred or thousand receptors), therefore their concentration is considered constant n . This means that

$$k = k_1 \cdot n \quad (4)$$

and therefore k is constant only in regions far from receptor saturation.

Since, however, a "reaction" between virion and cell can only occur if the virion has attached, equation (1) is actually a special case used for the description of those pseudo-first-order reactions, which occur on partially covered catalyzer surfaces of uniform activity.

$$-\frac{d[C]}{dt} = k_2 \cdot a \quad (5)$$

Equation (5) contains the Langmuir isotherm

$$a = a' \cdot \frac{[C]}{[C] + b} \quad (6)$$

where a is the adsorbate bound by unit catalyzer surface at constant temperature and a' is the boundary value of a at $[C] \rightarrow \infty$, where $[C]$ means the molar concentration of free adsorbate. Term b is a constant, depending only on the chemical characteristics of the interacting species and independent of the actual $[C]$.

If, and only if $[C] \ll b$, then

$$a = \frac{a'}{b} \cdot [C] = b' \cdot [C] \quad (7)$$

and equation (5) reduces to

$$-\frac{d[C]}{dt} = k_3 \cdot b' \cdot [C] \quad (8)$$

and

$$k_3 \cdot b' = \text{const.} \quad (9)$$

Briefly, constant k in equation (1) contains implicitly the condition that for a given system the parameters k_3 and b' are also constant.

Although experience has shown equation (1) to describe phenomenologically the process of initial phage-bacterium interaction, it is not necessarily

applicable to systems where one or another of the parameters lumped together into k may be non-constant.

As to points 2 and 3 of the classical interaction model referred to in the foregoing, it should be noted that the statement in equations (2) and (3) means that

$$\bar{V}_a = -\ln(1 - p_z) = f(p_z) = f^*(IC) \quad (10)$$

It might be of use to emphasize here that while $\bar{V}_a$ is a linear function of V_i , until $\bar{V}_a$ reaches its saturation level of $\sim 10^2$ to 10^3 , both p_z and IC are asymptotes and have their maxima at unity.

Let us set constant the time of interaction τ and replace $\bar{V}_a$ for V_i , then we have the following form of equation (1).

$$\frac{\bar{V}_a}{V_i} = 1 - e^{-k\tau} = K \quad (11)$$

and according to (10) also

$$\frac{f(p_z)}{V_i} = \frac{f^*(IC)}{V_i} = K \quad (12)$$

Clearly, equations (11) and (12) are valid only if all parameters included into K are constant. From equations (3), (10) and (12) it follows that in this case the probabilities of microscopic attachment p_a and infection p_i can only have the values

$$p_a = 1 \text{ or } p_a = \text{const.} \quad (13)$$

and

$$p_i = 1 \text{ or } p_i = \text{const.} \quad (14)$$

The actual value of p_i is determined by the incidence of competent cells in the system, μ and the infection producing collision probability p^* as

$$p_i = \mu \cdot p^* \quad (15)$$

This means either a complete lack of specificity or the existence of highly specific long-range and short-range interacting forces, rendering the virion-cell interaction immediate and irreversible on the first encounter. Since the virion-cell interaction is specific according to all experiences, we have to postulate that receptors satisfying the conditions just given are necessarily highly stable and persistent structures.

For the attachment of poliovirus to cells, both equations (1) and (11) were found to be valid. It was, however, shown that a stepwise increase of V_i and consequently of $\bar{V}_a$ caused a steep and irregular decrease of IC [1-3].

This suggested that, at least in this animal virus cell system, the probability of infection is not a simple function of the probability of attachment.

In order to understand the possible mechanism involved, we propose the following working hypothesis. Irreversible attachment may not require a full complementarity of interaction, while it may still render the virion sensitive to enzymatic decomposition. Full complementarity is, however, required for the specific interaction resulting first in conformational labilization and finally in opening of the capsid in a regulated manner. Such a process appears to be the precondition of the successful penetration or uptake into the cell of the undamaged viral genome. Actually, it has been shown that the major part of attached virions is rapidly inactivated at the cell's outer surface and only very few of the uniformly active virions succeed in initiating infection [4, 5].

Since the virion is a highly stable structure and the animal cell's membrane is metastable and highly dynamic, it appears reasonable to suppose that in addition to the state of competence [6, 7] some kind of receptor instability may also be responsible for the unusual behaviour of infection efficiency in function of V_i .

Several general and local membrane stabilizers were therefore examined for their possible yield-exalting effect in single viral cycles. Results of these studies for some representative types of compounds are shown in Table I.

Digitoxin, a specific inhibitor of K^+Na^+ ATPase appeared to be the most active in exalting the yield. It was in fact shown to exalt directly the IC and not the yield per cell [3].

It appeared that digitoxin not only exalted the IC but also stabilized its value at 0.45 at any value of $\bar{V}_a$ higher than the necessary minimum [3]. The pertinent numerical data are shown in Table II.

Table I

Relative yield-exalting activity of some general and local membrane stabilizers

Substance	mol wt	Maximum yeild exalting concentration, M*	Relative activity	Reference
Bovine albumin	6×10^4	3×10^{-5}	1	[8]
Arachidic acid	3.1×10^2	10^{-5}	3	[9]
Behenic acid	3.4×10^2	10^{-7}	300	[10]
Digoxin	7.8×10^2	10^{-8}	3 000	
Digitoxin	7.6×10^2	10^{-9}	30 000	[11]

* Activity was tested in single viral cycles in cells infected and maintained in suspension and the compound added at zero time of the cycle (for details see references)

Table II

Numerical data of attachment and infection efficiency at different free initial virion concentrations

V_i preset values	$\bar{V}_a$ calculated*	$\bar{V}_a$ experimental**	$IC = p_z$ calculated***	IC experimental****	IC_{di} experimental*****
0.125	0.103	0.106	0.096	0.050	0.080
0.250	0.207	0.221	0.182	0.075	n.d.
0.500	0.415	0.395	0.330	0.215	n.d.
0.750	0.622	0.580	0.452	0.270	0.42
1.000	0.830	0.832	0.551	0.300	0.48
1.250	1.036	0.966	0.633	0.280	0.45
1.500	1.245	1.172	0.714	n.d.	n.d.
2.000	1.660	1.725	0.827	0.230	0.45
2.500	2.075	1.875	0.865	0.124	n.d.
3.000	2.490	2.550	0.918	0.120	0.48
4.000	3.320	3.045	0.964	n.d.	n.d.
5.000	4.150	n.d.	0.982	0.100	n.d.
6.000	4.980	5.290	0.994	0.070	0.45

n.d. = not done

* from equation $\frac{\bar{V}_a}{V_i} = 0.83$

** determined as $V_i - V_t = \bar{V}_a$; average of at least 4 experiments

*** from equation $IC \cong p_z = 1 - e^{-\bar{V}_a}$

**** IC = untreated, IC_{di} = in presence of 10^{-8} M digitoxin; for details see reference [3]

As seen also from the data in Table II, of the equations (10) and (11), valid for the phage-bacterium model, only

$$\bar{V}_a = f(p_z) \quad (16)$$

was valid also here, while

$$p_z \neq IC \text{ and } p_z \neq IC \quad (17)$$

and also

$$\bar{V}_a = g(IC) \neq f^*(IC) \quad (18)$$

where g means a function other than f^* .

To find out the possible parameter(s) responsible for this fact, we analyzed in some detail certain known treatments of the interactions involving diffusion and attachment.

SMOLUCHOWSKY's treatment [12] has been applied to bacterial virion-cell systems by SCHLESINGER [13, 14] and DELBRÜCK [15]. VALENTINE and

ALLISON [16, 17, 18] claimed to have successfully adapted this treatment to animal virion-cell systems. However, as shown by KOCH [19] and OGSTON [20], their conclusions as to the possibility of estimating collision probability from attachment rate constant determination was erroneous. In fact, already the boundary condition set by SMOLUCHOWSKY [12] is extremely rigorous and actually means that an immediate irreversible attachment must occur at the first encounter between adsorbate and adsorbent:

$$C(r, t)_{r=r_0} = C_0 = 0 \quad (19)$$

where $C(r, t)$ is the free adsorbate concentration at t time at $r = r_0$ site, r being the radial distance and r_0 the adsorbent's radius. This implicitly means that the probability of successful collisions is always maximal ($p = 1$). The attachment rate constant is, however, maximal only if the transient term in SMOLUCHOWSKY's flux equation (20) becomes negligible.

$$\varphi(t) = 4\pi D r_0 C_\infty \left(1 + \frac{r_0}{\sqrt{\pi D t}} \right) \quad (20)$$

This means that

$$r_0 \ll \sqrt{\pi D t} \quad (21)$$

when

$$\varphi \cdot \frac{1}{C_\infty} = 4\pi D r_0 = K_{\max}. \quad (22)$$

where D stands for the sum of diffusion constants of the adsorbent and adsorbate and C_∞ is the initial, free adsorbate concentration at any r distance in the bulk phase at 0 time

$$C(r, 0) = C_\infty = \text{const.} \quad (23)$$

Clearly, for animal cells the transient term would become negligible only after several hours of attachment, therefore in such systems $K \neq K_{\max}$ even if $p = 1$. In fact, KOCH [19] demonstrated that in the proximity of $K_{\max}$, p is a rapidly changing function of K . Therefore, however precise the determination of K , it still fails, by principle, to allow for even the roughest estimation of p .

The above analysis of SMOLUCHOWSKY's treatment clearly shows that it cannot be used for solving our problem exposed in equations (16), (17) and (18).

This conclusion has lead us to try the treatment by COLLINS and KIMBALL [21] and COLLINS [22], representing a more general approach to the problem of diffusion-limited processes. They propose the boundary condition

$$C(r, t)_{r=r_0} = \gamma \cdot \left(\frac{\partial C}{\partial r} \right)_{r=r_0} \neq 0 \quad (24)$$

which also involves that $p \neq 1$. The term γ in equation (24) is related to the successful collision probability parameter like

$$\gamma = \frac{\langle s^2 \rangle}{\langle s \rangle} \cdot \frac{1}{p} \quad (25)$$

where $\langle s^2 \rangle$ and $\langle s \rangle$ are the mean radial square jump length and the mean jump length of the diffusing species in Brownian movement.

Now Fick's first law defines diffusional flux as

$$\varphi = D \cdot q \cdot (\nabla C)_q \quad (26)$$

where q stands for the adsorbent's surface and $(\nabla C)_q$ for the concentration gradient at the q surface. Under the initial and boundary conditions (23) and (24), the solution of (26) for spherical particles is

$$\varphi(t) = 4\pi DC_\infty \left\{ \left(\frac{pr_0^2}{pr_0 + \varrho} \right) + \frac{p^2 r_0^3}{\varrho(pr_0 + \varrho)} \exp \left[\frac{Dt(pr_0 + \varrho)^2}{(r_0 \varrho)^2} \right] \operatorname{erfc} \left[\frac{\sqrt{Dt}(pr_0 + \varrho)}{r_0 \varrho} \right] \right\} \quad (27)$$

where

$$\varrho = p \cdot \gamma = \frac{\langle s^2 \rangle}{\langle s \rangle} \quad (28)$$

and

$$\operatorname{erfc} x = \frac{2}{\sqrt{\pi}} \int_x^\infty e^{-z^2} dz \quad (29)$$

now if t is very large and r_0 very small, we have

$$\varphi(t) = 4\pi Dr'_0 C_\infty \left(1 + \frac{r'_0}{\sqrt{\pi Dt}} \right) \quad (30)$$

where

$$r'_0 = \frac{pr_0^2}{pr_0 + \varrho} \quad (31)$$

It follows therefore that if $p \neq 1$, we can obtain from equation (27) only a formal analogon (30) of SMOLUCHOWSKY's equation (20). Nevertheless, if $r'_0 \ll \sqrt{\pi Dt}$ then the transient term becomes negligible also in equation (30) and we have

$$\varphi = 4\pi DC_{\infty} \frac{pr_0^2}{pr_0 + \varrho} \quad (32)$$

Further, if r_0 is so small that $pr_0 \ll \varrho$, then

$$\varphi = 4\pi Dr_0 C_{\infty} \left(\frac{pr_0}{\varrho} \right) \quad (33)$$

which means that the flux is proportional to p . If, however, $pr_0 \gg \varrho$ then we have

$$\varphi = 4\pi Dr_0 C_{\infty} = \text{const.} \quad (34)$$

i.e. in this case the flux is constant and independent of p . Considering the restrictions made when deducing equations (32) and (33) from equation (27), it is clear that such restrictions cannot be realized in any experimental virion-cell system. Moreover, equation (24) does not mean anything else but the fact that $p = \text{constant}$.

Nevertheless, both SMOLUCHOWSKY's and COLLINS' treatments offer the possibility of considering the role and function of each microscopic parameter individually. Therefore, both are much more informative than the purely phenomenological equation (1), in which all these parameters are buried into the rate constant k .

In addition to the limitations already discussed, there are certain further restrictions to the use of SMOLUCHOWSKY's or COLLINS' treatment for the description of processes of virion-cell initial interaction. Both are based on Fick's second law, which is a second order partial differential equation with two variables.

$$\frac{\partial C}{\partial t} = D \nabla^2 C \quad (35)$$

where $\nabla^2 C$ is the divergence of gradient C . This involves that there is another boundary condition required which, although not mentioned specially, has been implicitly accepted in both treatments as

$$\lim_{r \rightarrow \infty} C(r, t) = C_{\infty} = \text{const.} \quad (36)$$

Equation (36) means that at infinite distance from the adsorbent, the concentration of free adsorbate is constant at any time (an open physical system). This condition is also one not realizable experimentally.

Now let us set the attachment time constant τ and use equation (20), then the particle count attached within τ time can be determined as

$$\Delta N = \int_0^{\tau} \varphi(t) dt = 4\pi D r_0 C_{\infty} \tau + 4\sqrt{\pi D} C_{\infty} r_0^2 \int_0^{\tau} \frac{1}{\sqrt{t}} dt \quad (37)$$

i.e.

$$\Delta N = 4\pi D r_0 C_{\infty} \left(\tau + \frac{2r_0}{\sqrt{\pi D}} \sqrt{\tau} \right) \quad (38)$$

Now in v volume of our system at t time, the particle count is

$$N = C_{\infty} \cdot v \quad (39)$$

At $t = \tau$ time the residual particle count in v will be $N - \Delta N$ and the average free particle concentration can be defined as

$$\bar{C}(\tau) = \frac{N - \Delta N}{v} \quad (40)$$

Let us have $v = 1$ and introduce the term $C(r, \tau)_{r=r_0} = \bar{C}_a(\tau)$ then we have

$$\bar{C}_a(\tau) = C_{\infty} - \bar{C}(\tau) \quad (41)$$

From equations (38) and (40) it follows that

$$\frac{\bar{C}_a(\tau)}{C_{\infty}} = 4\pi D r_0 \left(\tau + \frac{2r_0}{\sqrt{\pi D}} \cdot \sqrt{\tau} \right) = \text{const.} \quad (42)$$

According to COLLINS' treatment, we have the following general equation of flux

$$\varphi(t) = C_{\infty} f(t, p, D, \varrho, r_0) \quad (43)$$

where $f(t, p, D, \varrho, r_0)$ is a complex function of the parameters in the argument and parameters p, D, ϱ and r_0 are independent of time, initial concentration and concentration at t time of the free adsorbate. According to equations (41) and (43), we have

$$\bar{C}_a(\tau) = \int_0^{\tau} \varphi(t) dt = C_{\infty} \int_0^{\tau} f(t, p, D, \varrho, r_0) dt \quad (44)$$

Clearly the definite integral (44) is function only of τ and of the characteristic microscopic parameters of the system. Let us designate the definite integral as $F(\tau, p, D, \varrho, r_0)$, then after rearrangement of equation (44) we have

$$\frac{\bar{C}_a(\tau)}{C_{\infty}} = F(\tau, p, D, \varrho, r_0) = \text{const.} \quad (45)$$

Since virological systems are closed ones, *i.e.* attachment results in a decrease of the free adsorbate concentration, we have instead of (36) the boundary condition

$$\left(\frac{\partial C}{\partial r} \right)_{R,t} = 0 \quad (46)$$

which is the usual boundary condition for closed systems of spherical symmetry, where R is the radius of the outer boundary. Equation (46) actually means that particles neither enter, nor leave the system through the boundary.

Let us show that although the use of boundary condition (46) instead of (36) clearly affects the solution of equation (35), the validity of statement (45) is not affected. Namely, the partial differential equation (35) as well as the boundary conditions (24) and (46) are homogeneous. Therefore, if any $C^*(r, t)$ concentration function is a solution for the Fick II equation (35) and satisfies the boundary conditions (24) and (46), then function

$$C(r, t) = \lambda C^*(r, t) \quad (47)$$

where λ is a constant, is equally a solution for equation (35). The actual value of the λ parameter is determined by (23) mathematically inhomogeneous initial condition as

$$C(r, 0) = \lambda C^*(r, 0) = C_\infty \quad (48)$$

thus

$$\lambda = \frac{C_\infty}{C^*(r, 0)} = \frac{C_\infty}{K} \quad (49)$$

where K is constant. Now the flux according to (26) is

$$\varphi(t) = 4\pi D r_0^2 \left\{ \nabla C(r, t) \right\}_{r_0} \quad (50)$$

and from (48) also

$$\varphi(t) = 4\pi D r_0^2 \left\{ \nabla \lambda C^*(r, t) \right\}_{r_0} \quad (51)$$

thus, after rearrangement

$$\varphi(t) = 4\pi D r_0^2 \frac{C_\infty}{K} \left\{ \nabla C^*(r, t) \right\}_{r_0} \quad (52)$$

Equation (52) clearly shows that the solution of the problem with the experimentally realizable boundary condition (46) will have a form where C_∞ is a factor of multiplication, like in the SMOLUCHOWSKY (20) and the COLLINS (30) solutions. Thus

$$\varphi(t) = C_\infty h(t, p, D, \varrho, r_0) \quad (53)$$

where the $h(t, p, D, q, r_0)$ function contains, similarly to function (43), $f(t, p, D, q, r_0)$, only the microscopic parameters of the diffusion process. In view of the analogy of (43) and (53), the statement (45) is valid also for (54).

Thus, under experimentally realizable conditions, the only possible variable in function (45) is the successful collision probability parameter p . This parameter has the value of unity or is constant also in animal virion-host cell systems, as shown in Table II. Since, however, for these systems the equations (16), (17) and (18) are valid, the difference between bacterial and animal virion cell systems appears to be linked with the diffusion unrelated parameters μ and p^* , ($p_i = \mu \cdot p^*$).

Under standardized experimental conditions, the value of μ can be regarded as practically constant for any given cell population. Therefore, if p^* and consequently p_i are constant, changes of IC in function of V_i should follow a saturation-type curve. Since this is the experimental observation in the digitoxin-treated system, in this case the probability of collision leading to infection has become constant and independent of the actual V_i , provided the latter is above a certain minimum. If the curve describing changes of IC in function of V_i is not of the saturation type, then p_i is non-constant and depends on V_i . This means that either μ or p^* or both are variable.

Since in our experimental system p_i was rendered constant by the addition of a local membrane stabilizer, we propose the following hypothesis. Cellular receptors for polio virions are not stable structures, but only transient conformational states of certain regions of the cell membrane. The apparent constant average receptor count per cell n means only that the incidence of the receptor conformation per cell is statistically constant at any time. This does not contradict the possibility that the localization and time of existence of this conformation at any given site is subject to random variations. Therefore, the chance of a virion to hit a randomly oscillating target is *a priori* poor. If a skew hit results in irreversible, but quasi complementary attachment, a perturbation occurs and the chance for a good hit by another virion is further diminished.

Digitoxin stabilizes the Na^+K^+ ATPase in one of its possible conformational states. This results by some direct or indirect intra-membranal interactions in the stabilization of the receptor conformation for polio virions too. Thus n will be not only statistically constant, but also the localization and the complementary pattern of the receptors becomes stable. The chance for a virion to hit successfully such a receptor is fairly good, as seen also in bacterial virion cell systems. Therefore, in such a case the main parameter controlling the efficiency of infection will be the actual ratio of competent cells in the population. As shown by EREMENKO *et al.* [6, 7], the competence of cells to replicate poliovirus is highly variable in the different phases of their life cycle. Since we have worked with non-synchronized cells, the $IC = 0.45$ value ob

tained in digitoxin-treated systems may be accepted as a reasonable average for the ratio of competent cells in the population.

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A HIGHLY SPECIFIC PHAGE ATTACKING BACILLUS ANTHRACIS STRAIN STERNE

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Summary. Temperate phage AP 50 isolated from a soil specimen failed to act upon different species of *Bacillus* except two *Bacillus anthracis* strains showing a moderate sensitivity. In host range, antigenic structure, UV, pH and heat resistance it differed from other *B. anthracis* phages. In electron micrographs phage AP 50 was characterized by a double-layer coat on the hexagonal head. The phage was inactivated by chloroform and ether.

The present study deals with a phage which, unlike a number of phages attacking *Bacillus anthracis* [1, 2], is highly specific in host range.

Materials and methods

Culture medium. Yeast-extract-peptone (YP) medium [3] solidified with 1.5% agar was used. The phage was diluted with saline containing YP broth at a proportion of 5: 1.

Bacterial strains. Acapsulogenic *B. anthracis* strains CN 18–74 and CN 35–18 of STERNE, [4], streptomycin resistant strain (CN 18–74/Str) obtained from CN 18–74 by one-step mutation and strain CN 18–74 *cur* not inducible with mitomycin C [5] were used. As indicator cultures, 16 *B. anthracis* strains (Table I), 12 *B. cereus*, 5 *B. megaterium* and 5 *B. subtilis* strains were employed.

Phages. *B. anthracis* phages 27cr, 27tl, 29cg [1, 2] and the clear mutant of phage W [6] were used.

Isolation of phages was performed from soil specimens with the streptomycin method [1]. The phages were propagated on strain CN 18–74 Str, then processed according to IVÁNOVICS and LANTOS [1]. From the enriched phage material, temperate phage AP 50 was isolated by the soft agar method on culture CN 18–74 Str. From the original isolate yielding turbid plaques, a clear mutant (AP 50 C) was derived by nitrosoguanidine treatment.

Phage titration was carried out with the soft agar method [7] using strain CN 18–74 *cur* which was assumed to be free from defective phages [5].

Host range was determined by spotting 10-fold serial dilutions of membrane filtered phage material on soft agar layer inoculated with the indicator strain.

Heat sensitivity. The phage was diluted 1: 100 with pH 7.2 buffer, kept in water bath at the required temperature then the number of survivors was determined.

pH sensitivity. To 0.5 ml phage 0.2 M acetate (pH 3.6, 4, 4.6, 5, 5.6) and Tris-maleinate (pH 7.6, 8, 8.6) buffer series were added. The mixtures were incubated at 37°C and sampled at intervals for the number of survivors.

UV inactivation was performed with a Hanau germicidal lamp: the energy of irradiation was measured photometrically as described by LATARJET *et al.* [8]. The filtered phage was diluted 1: 100 with 0.2 M Tris-maleinate buffer (pH 7.2) and irradiated at 26 erg/cm²/s in 5 ml amounts in 10 cm Petri dishes rotated horizontally throughout irradiation then tested for survival.

Phage adsorption. Strain Vollum C- (10⁷ p.f.u./ml) was infected in liquid medium with 0.1 m.o.i. AP 50. After shaking at 37°C for 60 and 120 min, the degree of adsorption compared to the control was examined [7].

Immune serum was prepared in rabbit by intravenous injection of phage purified by cyclic ultracentrifugation. The K value was estimated according to ADAMS [7].

Purification and concentration of phage. Three to 5 litres YP broth distributed in 200 ml portions into 1 litre Erlenmeyer flasks were inoculated with strain CN 18-74 *cur*. The cultures were incubated overnight. At OD 0.4 (Bausch-Lomb spectrophotometer, 620 nm), corresponding to approximately 10^8 bacteria per ml [5], the culture was infected with $5 \times$ m.o.i. AP 50 c phage. The lysates were centrifuged at 5000 g, then concentrated with 5% polyethylene glycol 6000 and 0.5 M NaCl as described by YAMAMOTO *et al.* [9]. After centrifugation at 5000 g for 30 min the deposit was resuspended in a one-hundredth volume of the original amount of

Table I
Sensitivity of B. anthracis strains to phage AP 50

Strains	Highest effective dilution of phage AP 50
Vollum C-	×
Davis	×
NPA C-	—
68 C-	10^{-3}
77 C-	—
81 C+	10^{-3}
87 C+	—
88 C+	—
89 C+	×
91 C+	×
92 C+	—
93 C+	—
94 C+	—
96 C+	—
Sterne CN 18-74	10^{-9}
Sterne CN 35-18	10^{-9}

× Semi-clear spot with undiluted phage

C- acapsulogenic

C+ capsulogenic

buffer containing 0.05 M Tris HCl (pH 7.2), 0.01 M magnesium acetate, 0.11 M ammonium acetate and 0.001 M mercaptoethanol (TMAM buffer). After cyclic centrifugation in the Janetzki Vac 60 ultracentrifuge at 8000 g for 10 min and 30,000 g for 90 min the phage was centrifuged in preformed sucrose density gradient (60–20%) in the Janetzki SW₃₀ rotor at 50,000 g for 180 min. In this manner the purified phage appeared as a sharp band. After having drilled the bottom of the tube, the fractions dripping out were collected. Fractions with high titre were united and dialysed against TMAM buffer. After this procedure 46% of the original particle counts was recovered. When phage AP 50 (temperate) was used, the same procedure was applied, except that after phage infection the culture was induced at OD 0.2 with mitomycin C (0.5 µg/ml), then reincubated until lysis occurred.

Electron microscopy. Negative staining with 2% uranyl acetate was used [10]. The purified phage was mounted on LKB grids covered with Formvar film; after 60 s adsorption the excess liquid was removed with filter paper and the preparation was stained. For electron microscopy, a Yem 100B apparatus operating at 80 kV was used.

Results

Plaque morphology. Phage AP 50 produced very turbid plaques 1–2 mm in diameter on indicator strain CN 18–74 Str. The phage titre was usually 10^{10} p.f.u./ml. A similar plaque morphology and e.o.p. value was obtained with strains CN 18–74, CN 35–18 and CN 18–74 *cur*.

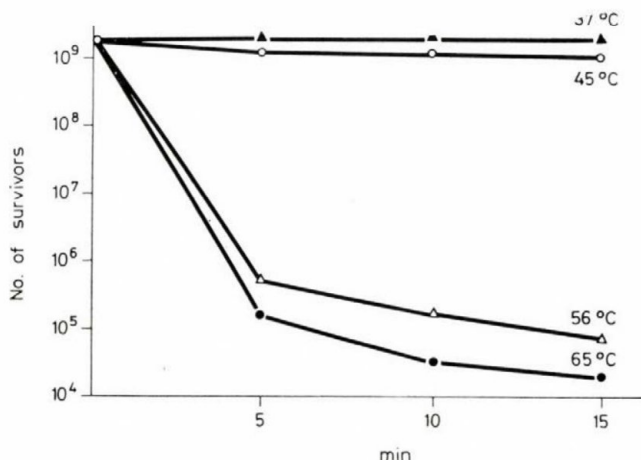


Fig. 1. Heat inactivation of phage AP 50

Host range. Phage activity was observed only against two *B. anthracis* strains (68 C⁻ and 81 C⁺) in addition to the two STERNE strains (Table I). The phage failed to act upon a number of other *Bacillus* cultures. With the moderately sensitive strains 68 C⁻ and 81 C⁺ a low titre phage was obtained in cultures shaken at 37°C and induced with mitomycin C (10^3 p.f.u./ml). Four *B. anthracis* cultures produced a semi-clear spot when undiluted phage had been pipetted on them. Young liquid medium subcultures prepared from the corresponding sites were induced with mitomycin C; after reincubation the culture showed no definite lysis and the supernatant obtained after centrifugation failed to act on the homologous strain but exhibited a titre corresponding to the dilution of the phage on the Sterne strains. This finding is in agreement with the observation that an adsorption of phage AP 50 on strain Vollum C⁻ could not be demonstrated.

Heat sensitivity of phage AP 50. Heat inactivation curves are presented in Fig. 1. At 56°C and at 65°C the phage titre decreased by 3.5–4 exponents within 15 min. Phage AP 50 behaved similarly to other *B. anthracis* phages, as at higher temperatures the number of survivors decreased less sharply after an initial exponential decline. Phage AP 50 was fairly stable on storing at

4°C: there was no considerable decrease in titre after several months. At 24°C an 80% inactivation was observed after 12 days.

pH sensitivity. Phage AP 50 was fairly stable between pH 5 and 8.6. Under pH 5 the number of survivors decreased rapidly (Fig. 2).

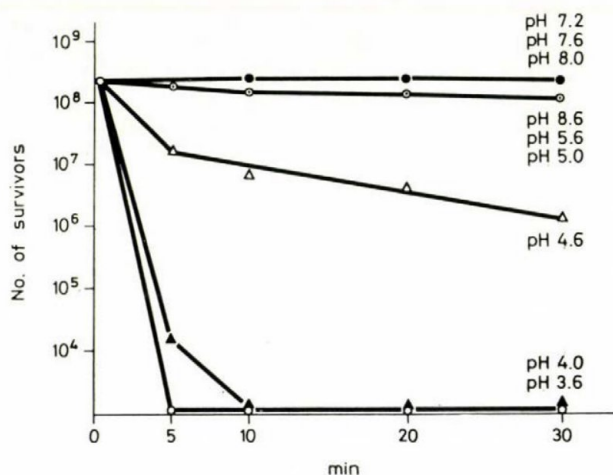


Fig. 2. pH sensitivity of phage AP 50

UV sensitivity. As compared to the control, for 99% inactivation 2500 erg/m² was needed (Fig. 3). This value reflects a rather high UV resistance as compared to other *B. anthracis* phages examined previously [2].

Antigenic structure of phage AP 50. Relationship of phage AP 50 to *B. anthracis* phages isolated by IVÁNOVICS and LANTOS [1] and to phage W of McCLOY [6] was examined. The immune serum prepared against AP 50 neutralized 90% of the homologous phage particles at 1 : 1600 dilution ($K = 98$) in 20 min. The same serum failed to neutralize at 1 : 10 dilution in 60 min the above *B. anthracis* phages representing three different antigenic structures.

Sensitivity to organic solvents. The titre of concentrated AP 50 phage was considerably decreased after vigorous shaking in 25% ethyl ether and in 5% chloroform (Table II). Without shaking the decrease was somewhat less.

Table II

Inactivation by chloroform and ether of concentrated purified phage AP 50

Treatment	Titre (p. f. u./ml)
Nil	1.5×10^{11}
5% chloroform 30 min	1.7×10^7
25% ethyl ether, 30 min	1.3×10^7

Electron microscopy failed to show any morphological difference between the original isolate and its clear mutant. Both phages were characterized by a strikingly high number of degraded tailless virions. There was a 54% p.f.u. difference between the native lysate and the purified, concentrated phage material, indicating that during the purification procedure more than half of

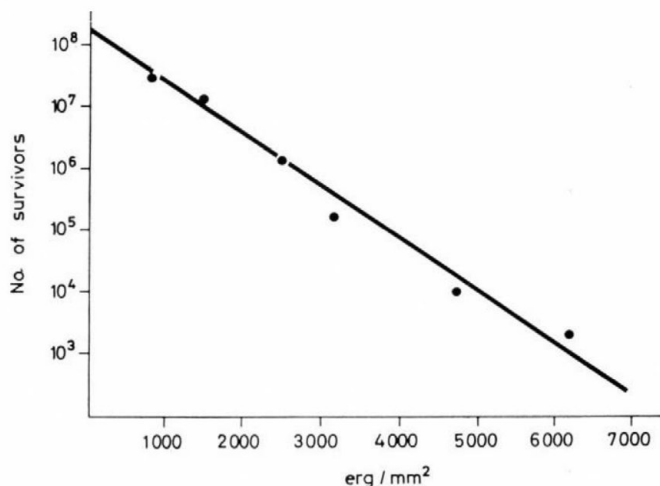


Fig. 3. UV inactivation of phage AP 50

the particles had lost infectivity. The electron micrographs suggested that the original lysate contained great numbers of degraded virions that had lost their nucleic acids: such forms occurred in more than 50% in the concentrated, purified phage material Fig. 4 (a and b).

The diameter of the hexagonal head of the intact phage was 80 nm. The non-contractible tail was 80 nm in length and 13 nm in width. The tail of the intact phage was solid without cross-striation. Especially in degraded virions, the head had a relatively thick (16 nm) coat. In Fig. 4 (c) the coat seems to have two layers, the inner one is thicker (approx. 8 nm), the outer one is thinner (approx. 5 nm). In intact phages the outer layer seems to cover the central particle (see Fig. 4a and b).

Discussion

Phage AP 50 exhibits unusual properties. First of all, it has a considerable host range specificity. Both the original isolate and its mutant (AP 50 C) could be propagated well on acapsulogenic STERNE strains used for their isolation. Against other *B. anthracis* strains they were slightly or not at all active. These strains have probably no receptors for phage AP 50; this is supported

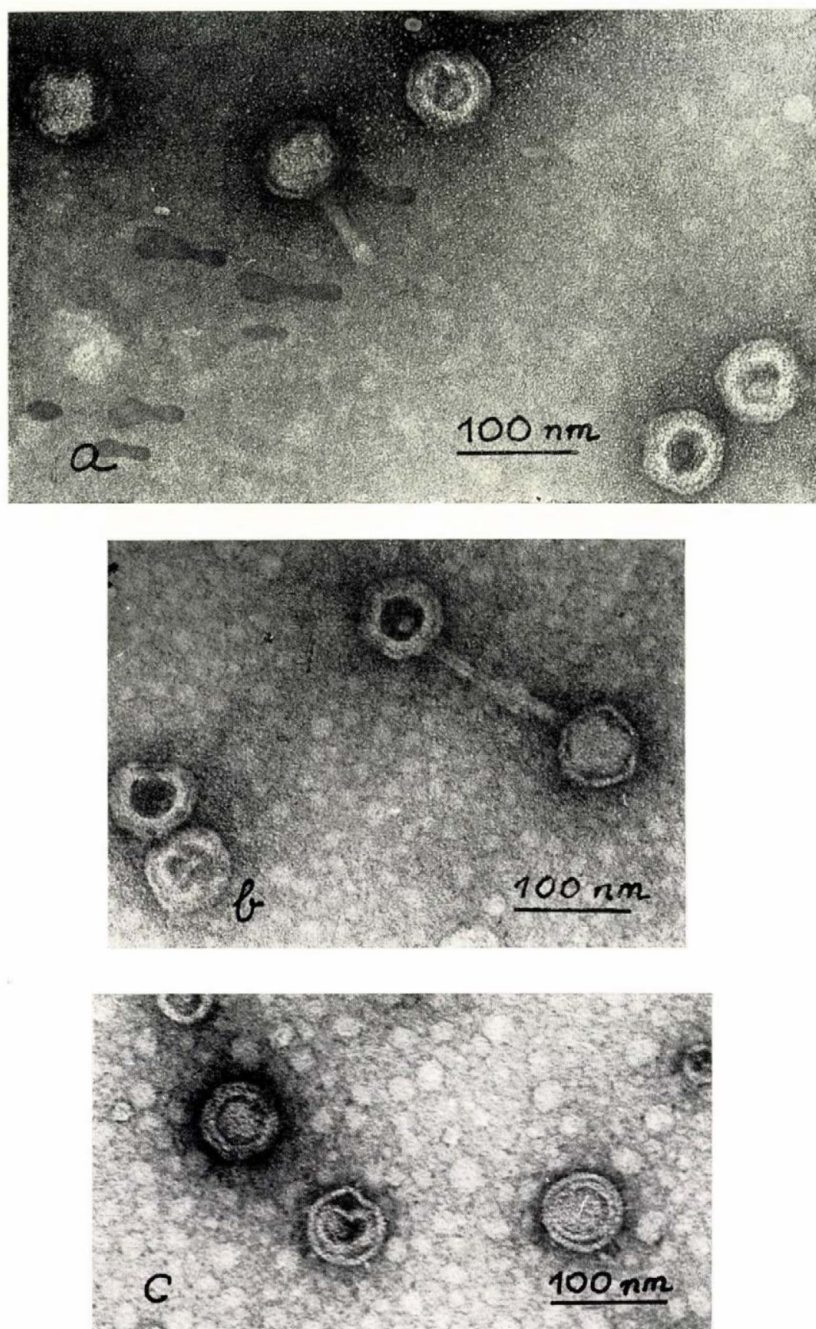


Fig. 4 (a, b and c). Electron micrograph of purified and concentrated phage AP 50. Uranyl acetate staining ($\times 150,000$)

by the finding that adsorption experiments on strain Vollum C⁻ gave negative results.

LANTOS *et al.* [2] included *B. anthracis* phages into 3 antigenic groups. The groups could be distinguished by UV, heat and pH resistance. In view of the above criteria, phage AP 50 belongs to none of these groups.

Electron microscopy showed that phage AP 50 with its hexagonal head and relatively short, noncontractible tail, belongs to group C of BRADLEY [11]. TIKHONENKO [12] classified such phages into group III (short-tailed phages).

Phage AP 50 differs from other phages in its high sensitivity to chloroform and ether and also in having a definite double-layer coat on its head. Thus it may be assumed that the coat contains not only proteins but also lipids. ESPEJO and CANELO [13, 14] and VIDAVER *et al.* [15] described similar lipid-containing, organic solvent sensitive phages (PM2, ϕ 6), and these phages were also characterized by a double-layer coat. However, they act upon Gram-negative bacteria, while AP 50 can be propagated on a Gram-positive organism. In view of its unusual behaviour, phage AP 50 seems to be of interest for further studies.

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METABOLIC EFFECT OF INTERFERON PREPARATIONS AND SOME INTERFERON INDUCERS: ACTIVATION OF ADENYLATE CYCLASE SYSTEM

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Summary. Crude or purified mouse interferon preparations activate adenylate cyclase and raise the level of 3'5'-cAMP in L cells, though this activity does not parallel the antiviral capacity of the preparations. PolyI.polyC and polyA.polyU also activate adenylate cyclase and the effect seems to be correlated with the molecular size of the polymers. DEAE-dextran, a polycationic substance proved to be an effective activator of adenylate cyclase. The effect of the inducers and activators administered simultaneously seems to be additive.

It has been reported previously [1] that substances activating adenylate cyclase and influencing the intracellular level of cAMP, may regulate the establishment of antiviral state (AVS) of cells and the induction and/or production of interferon (IF). This phenomenon resembles that found with "priming", a particular action of IF [2–8]. It was therefore of interest to study whether the adenylate cyclase system was activated by IF preparations and some IF inducers.

Materials and methods

Mouse L-929 cells were maintained in Parker 199 medium supplemented with 5% bovine serum. Crude IF was the serum of CFLP mice injected either by polyI.polyC (Reanal Fine Chemicals, Budapest) or Semliki forest virus (brain preparation of baby mice) Kumba serotype (SFV), in doses of 10 µg and 10⁸ p.f.u. per mouse, respectively. Purified IF with a specific activity of 300 000 units/mg protein was a gift of Dr. G. Bodo (Arzneimittel Forschungs-Institut, Vienna).

Complete monolayers of L cells grown in 60 mm Petri dishes in 5% CO₂ atmosphere were used in the experiments. For determining cAMP levels the cells were labelled with [³H]adenine for 24 hr according to SHIMIZU *et al.* [9]. IF preparations or other substances diluted in serum-free Parker 199 medium were added in 1 ml volume. Control cells received 1 ml serum-free medium only. The samples were incubated at 37°C for 1 hr and then washed with saline and scraped from the glass. The cells were immediately taken up in 1 ml of recovery mixture containing 1000 µg ATP and 100 µg 3'5'-cAMP, disrupted by freezing-thawing, then deproteinized in boiling water for 3 min. After centrifugation at 800 g for 5 min the supernatants were put on Whatman MM paper, chromatographed at room temperature 3 × 4 hr in a mixture of butyl alcohol–acetone–ammonia–acetic acid–water (90 : 30 : 20 : 20 : 40) and the 3'5'-cAMP separated was determined in a (PPO, POPOP, Toluene) Packard Tricarb scintillation spectrometer.

The values yielded were expressed as cpm/mg protein, and corrected by factors obtained with the use of the recovery mixture.

Results and discussion

Data in Table I show that crude mouse IF preparations induced either by SFV or polyI. polyC activated markedly the adenylate cyclase system in L cells or alternatively, inhibited the activity of cAMP phosphodiesterases, as compared to the control. Pooled mouse serum, free of IF activity, had also some effect. In contrast, purified IF showed a faint effect only, which was not proportional with the antiviral activity of the preparation. PolyI. polyC and polyA. polyU preparations activated also adenylate cyclase in L cells [9], as shown in Table II, and their activity seemed to vary with the molecular size of the polynucleotides.

Table I

Activation of adenylate cyclase in L cells by crude and purified interferon preparations

		3'5'-cAMP content in % of control $\times$ 100 (cpm/mg protein)
Control		1.0*
Mouse serum		1.4**
Interferon — SFV	100 units	2.2**
Interferon — I : C	100 units	2.1**
Interferon — I : C	10 units	1.3***
Purified interferon	30 units	1.1****
	300 units	1.3
	3000 units	1.5
	30,000 units	1.8

L-929 cells were grown, in 60 mm Petri dishes and labelled with [^3H] adenine for 24 hr. The complete monolayers (ten plates of each group) were washed with saline and treated with various IF preparations diluted in serum-free medium at 37°C for 1 hr. The radioactive 3'5'-cAMP formed was determined in the deproteinized cell sap and expressed as cpm/mg protein corrected by factors obtained by use of recovery mixture. Control: 800 cpm/mg protein; * mean $\pm$ S.E. (0.12), ** serum diluted 1 : 10; *** serum diluted 1 : 100; **** 300.000 units/mg protein specific activity

Prostaglandin E_1 (PGE_1) and isoproterenol (IPR), as expected, and surprisingly, DEAE-dextran (DEAE-dx) activated very efficiently adenylate cyclase as well.

Furthermore, DEAE-dx [10], [11], PGE_1 , IPR, crude IF and mouse serum preparations, administered together with polyI. polyC produced an increased activity and their effects seemed to be roughly additive. This is in accord with our earlier observations [1] and indicate that substances increasing the intracellular level of 3'5'-cAMP potentiate the biological as well the biochemical activities of IF and IF inducers.

Table II

Activation of adenylate cyclase in L-cells by several activators and interferon inducers

		3'5'-cAMP content in % of control $\times 100$ (cpm/mg protein)
Control		1.0*
DEAE-dx	20 $\mu\text{g/ml}$	4.6
IPR	25 $\mu\text{g/ml}$	3.5
	2.5 $\mu\text{g/ml}$	1.4
PGE ₁	5 $\mu\text{g/ml}$	1.6
I : C (S _{w20} : 16)	10 $\mu\text{g/ml}$	2.6
I : C (S _{w20} : 6)	10 $\mu\text{g/ml}$	1.4
A : U (S _{w20} : 6)	10 $\mu\text{g/ml}$	1.3
I : C (S _{w20} : 16)	10 $\mu\text{g/ml}$	
In combination with		
DEAE-dx	20 $\mu\text{g/ml}$	5.6
IPR	25 $\mu\text{g/ml}$	4.4
PGE ₁	5 $\mu\text{g/ml}$	3.9
Mouse serum	1 ml	2.9**
Interferon — I : C	1 ml (100 U)	3.4**

Control: 860 cpm/mg protein; * mean $\pm$ S.E. (0.12); ** serum diluted 1 : 10; for further details see Table I

Whether IF or some impurity present in the IF preparations is responsible for the observed activation of adenylate cyclase, remains to be studied. Nevertheless, it is tempting to suggest that properties other than the antiviral capacity of IF and some IF inducers, *e.i.* the "priming" [2-8], immune-enhancing or immuno-suppressive [12-17], anti-tumour [18-21], anti-inflammatory [22] effects, etc., are attributed to or accompanied with the activation of the adenylate cyclase system.

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EFFECT OF TRYPSIN AND ANTIPENTON SERUM ON THE INTERFERON INDUCING ABILITY OF HUMAN ADENOVIRUSES

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Summary. Trypsin treatment of types 3, 5, 6, 8 and 12 of human adenovirus abolished or decreased considerably their interferon inducing capacity in chick embryo fibroblast cells. Antipenton sera against human adenovirus types 5 and 8 inhibited the interferon induction by homologous types, but failed to affect their infectivity. Interferon induction by heterologous types from the same and other subgroups was not influenced. These data confirmed our earlier assumption that the penton antigen is involved in the induction of interferon by human adenoviruses in chick embryo fibroblast cells.

Formation of interferon by several types of human adenovirus in chick embryo fibroblast cells has been observed in this laboratory. As trypsin treatment abolished the interferon inducing capacity of types 5 and 12 of human adenovirus, the role of penton in the induction was suggested [1]. This was supported by data obtained with heat and UV irradiated virus. Adenoviruses heated at 56°C for a few minutes failed to stimulate interferon production. In contrast, virus inactivated by UV irradiation effectively stimulated interferon production in chick cells [2].

This paper deals with experiments in which the effect of trypsin-treatment and that of the antipenton and antihexon sera on several human types regarding their interferon inducing ability in chick fibroblast cells was studied.

Materials and methods

Viruses. Human adenovirus types 5 and 12 were kindly provided by Dr. K. KÖHLER (Max Planck Institute, Tübingen). Types 10 and 27 were kindly supplied by Dr. R. WIGAND (Homburg, Saar) and types 2, 3, 6, 13 and 16 by Dr. H. G. PEREIRA (National Institute for Medical Research, London). Type 8 was isolated in this laboratory [3]. Adenoviruses were propagated in HEp-2 cells maintained in Gey's medium which contained 5% rabbit serum, 0.25% lactalbumin hydrolysate and 252 µg/ml arginine.

Sindbis virus obtained through the courtesy of Dr. I. GRESSER (Boston) was used for challenge. The virus was grown in primary chick embryo fibroblast cells cultured in Gey's medium containing 5% calf serum and 0.25% lactalbumin hydrolysate.

Infectivity titration. Infectivity of adenovirus was determined in test tube cultures of HEp-2 cells with the dilution method. The medium was renewed every 4 or 5 days. The TCID₅₀ was calculated after 4 weeks of inoculation by using the formula of REED and MUENCH [4].

Infectivity of Sindbis virus was determined by plaque assay of PORTERFIELD [5] with some modifications [6].

Treatment with trypsin. After incubation with trypsin (twice crystallized, Serva) at 1 mg/ml concentration in a water bath at 37°C for 60 min, the virus sample was placed in an ice bath and a double amount of soybean trypsin inhibitor (Serva) was added.

Preparation of capsid components. The crude virus was purified with fluorocarbon and ultracentrifugations. Virus antigens were separated by repeated anion-exchange chromatography, density gradient ultracentrifugation, erythrocyte adsorption and elution, and gel-filtration on Sephadex G-200 columns as described earlier [7, 8].

Preparation of immune sera. The sera against penton and hexon of types 5 and 8 were prepared in rabbits as described previously [8].

Interferon production. Two-day-old monolayers of chick embryo fibroblasts were infected with 0.1 ml of the appropriate virus inoculum. When serum-treated virus was tested, the undiluted virus was mixed with an equal volume of 1 : 2 diluted immune or normal rabbit serum. The mixtures were kept at 37°C for 2 hr and then 0.2 ml aliquots were inoculated onto monolayers of chick embryo fibroblast cells. After 2 hr adsorption, 5 ml of fresh medium were added to each culture. The fluids were harvested and pooled after incubation at 35°C for 48 hr, then centrifuged at 3000 rpm for 15 min. After heating at 56°C for 1 hr the supernatant was kept at 4°C until tested for interferon activity.

Interferon assay was performed on chick embryo fibroblast monolayers by the plaque-reduction method as described earlier [2].

Results

Treatment of adenoviruses with trypsin destroyed or diminished their ability to induce interferon (Table I) without affecting their infectivity.

Table I
Interferon induction by trypsin-treated human adenoviruses

Virus type	Interferon titre induced by	
	untreated virus	trypsin-treated virus
3	16	< 4
5	64	< 4
6	16	< 4
8	256	4
12	1024	8

The results of three independent experiments showed that type 8 antipenton sera inhibited the interferon inducing capacity of human adenovirus type 8 in chick embryo fibroblast cells; a neutralizing effect on infectivity was not observed (Table II). On the other hand, type 8 antihexon serum inhibited both interferon induction and infectivity. Of two antipenton serum preparations tested (rabbits No. 133 and 134) one was more effective in neutralizing the interferon inductive capacity of type 8.

Type 8 antipenton serum was tested against heterologous types too (Table III). This antipenton serum inhibited only the interferon by the homologous type and had no effect on the interferon induction by types 10, 13, 27 from the same subgroup and on that of types 2, 5, 6, 12 and 16 from the other two subgroups.

Table II*Interferon induction and infectivity of human adenovirus type 8 after treatment with immune sera*

Experiment No.	Rabbit serum	Interferon titre	Virus titre log ₁₀ LD ₅₀ /0.1 ml
1	Normal	128	NT*
	Antipenton (133)**	4	NT
	Antihexon	< 4	NT
2	Normal	256	1.5
	Antipenton (133)	8	1.5
	Antipenton (134)	16	1.5
	Antihexon	< 4	< 1.5
3	Normal	256	1.5
	Antipenton (133 and 134)	32	1.5
	Antihexon	< 4	< 1.5

* Not tested

** Designation of rabbit

Table III*Effect of adenovirus type 8 antipenton serum on the interferon inducing capacity of different human types*

Adenovirus		Titre of interferon induced by	
Sub-group	Type	untreated virus	serum-treated virus
1	16	32	32
2	8	64	< 4
	10	64	32
	13	64	64
	27	64	32
	2	16	16
3	5	32	32
	6	16	16
	12	256	128

The effect of antipenton serum prepared against type 5 was also studied on interferon induction by homologous and heterologous types (Table IV). It had a blocking effect on the homologous type whilst no influence on heterologous types belonging to any subgroup.

The penton antigen of adenoviruses has been known to be sensitive to trypsin [9, 10] but resistant to UV irradiation [9]. RUSSELL *et al.* [11] found

Table IV

Effect of adenovirus type 5 antipenton serum on the interferon inducing capacity of different human types

Adenovirus		Titre of interferon induced by	
Sub-group	Type	untreated virus	serum-treated virus
1	16	8	8
2	8	64	64
3	5	16	< 4
	6	64	64
	12	256	256

that heating of adenovirus type 5 at 56°C resulted in a specific disintegration of the penton base followed by the liberation of the surrounding five hexons.

Trypsin treatment destroyed the interferon inducing ability of any adenovirus type tested in chick embryo fibroblast cells suggesting the role of penton in the induction of interferon. This was in agreement with results of experiments in which the effect of heat and UV on the interferon inducing ability of human types was tested [2]. The antipenton serum prevented interferon induction by the homologous type without affecting its infectivity, confirming the hypothesis that the penton antigen has a decisive role in the induction of interferon by adenoviruses in chick embryo fibroblast cells.

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ELECTRON MICROSCOPIC STUDY OF HUMAN ADENOVIRUSES WITH DIFFERENT INTERFERON INDUCING ABILITY

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Summary. Human adenovirus types 6, 8, 12 and 16 were found to induce different amounts of interferon in chick embryo fibroblast cells. Types 8 and 12 were more effective than types 6 and 16. The interferon inducing ability did not depend on the multiplicity of infection. The possible correlation between the non-viral paracrystal forming and the interferon inducing abilities of types 6, 8, 12 and 16 has been studied. The events in virus infected HEP-2 cells were observed by electron microscopy. Different morphological changes have been observed during the replication of different types. The effective interferon inducers failed to stimulate the formation of non-viral paracrystalline structures, which were demonstrated only in type 6 infected HEP-2 cells. These data suggest that the interferon inducing capacity of human adenoviruses does not depend on their ability to stimulate para crystals during replication

Formation of non-viral paracrystalline structures has been demonstrated in the nuclei of cells infected with some strains of different adenovirus types [1–4].

It has been observed previously that a trypsin sensitive protein was responsible for the interferon induction by adenoviruses, and different human types exhibited different interferon inducing ability [5, 6]. These facts have promoted us to study whether non-viral paracrystals formed during replication influenced the interferon inducing capacity of the strains.

The present paper described experiments in which the correlation between the formation of paracrystalline structures during replication in HEP-2 cells and the induction of interferon in chick embryo fibroblast cells by human adenovirus types 6, 8, 12 and 16 has been studied.

Materials and methods

Viruses. Human adenovirus serotypes 6 and 16 were kindly supplied by Dr. H. G. PEREIRA (National Institute for Medical Research, London). Type 8 was isolated from a patient suffering from keratoconjunctivitis [7]. Adenovirus type 12 was kindly provided by Dr. K. KÖHLER (Max Planck Institute, Tübingen). The viruses were propagated in HEP-2 cells cultured in Eagle's basal medium containing 10% inactivated calf serum and 0.3% tryptose phosphate broth according to MACPHERSON and STOKER [8]. After virus infection the cells were maintained in Gey's solution enriched with 5% rabbit serum and 0.25% lactalbumin hydrolysate. The viruses were liberated by freezing and thawing the cells five times, then cell debris was removed by centrifugation at 3000 rpm for 15 min.

Leukocyte cultures were prepared from the blood of 6–12-month-old chicken as described previously [9]. Mixed leukocyte cultures obtained from 5–6 chicks were used in each

experiment. Leukocytes were diluted to the proper concentration in Eagle's basal medium containing 10% tryptose phosphate broth and 20% calf serum. One ml samples containing 10^7 leukocytes were used per culture.

Infectivity titrations. Infectivity assays were made in test tube cultures of HEp-2 cells. Serial tenfold dilutions of the virus were prepared in Hanks's balanced salt solution and three cultures were inoculated with each dilution. The medium was renewed every 4 or 5 days and the cells were examined for cytopathic effect over a 4-week period and then the infective titre was calculated as TCID₅₀ using the method of REED and MUENCH [10].

Production of interferon in chick embryo fibroblast cells. The method for preparing chick embryo fibroblast cell cultures and the medium used were described earlier [6]. Petri dishes (60 mm in diameter) were seeded with 2.5×10^7 primary chick embryo fibroblast cells. Two days later the medium was removed and 0.1 ml of the virus was introduced into two cultures. After 1 hr adsorption, 5 ml of fresh medium was added to each culture and incubated at 35°C for 48 hr. The fluids were harvested and pooled, then centrifuged at 3000 rpm for 15 min. The supernatants were kept at 4°C until tested for interferon activity.

Production of interferon in leukocytes. Undiluted virus was added at 0.2 ml aliquots to the leukocyte cultures. The cultures were incubated at 36°C. After 48 hr of incubation the culture fluids were harvested and centrifuged at 3000 rpm for 15 min. The supernatants were stored at 4°C until tested for interferon activity.

Interferon assays were performed as described previously [6]. Sindbis virus was used as challenge virus. The samples to be tested for interferon activity were heated at 56°C for 1 hr. The interferon titre was expressed as the reciprocal of the dilution causing a 50% depression of the control plaque count.

Electron microscopy. The tissue cultures were cooled to 4°C for 5 min and the cells were fixed *in situ* with 1% phosphate-buffered glutaraldehyde at pH 7.3 for 20 min. After fixation the cells were scraped off the glass surface with a rubber policeman, pelleted gently, and the pellet was washed 7 times with Millonig phosphate buffer pH 7.3 containing 0.54% glucose. After washing postfixation was carried out with 1% osmium tetroxide in the same buffer at pH 7.3 for 4 hr. The pellet was repeatedly centrifuged and washed twice with the above mentioned buffer, and dehydrated in ethanol. While dehydrating, the pellet was kept standing overnight in saturated uranyl acetate solution of 70% aqueous alcohol, then embedded in araldite. The thin sections were prepared using an LKB ultramicrotome "Ultratome 80" and glass knives and were stained with lead citrate for 10 min. The pictures were taken with a JEOLCO JEM Type 100B electron microscope, with the use of 80 Kv and an objective aperture of 40 μ .

Results

Interferon induction by types 6, 8, 12 and 16 in chick embryo fibroblast cells. Chick embryo fibroblast cells were inoculated with types 6, 8, 12 and 16 at different multiplicities. The titres of interferon induced are summarized in Table I.

Types 8 and 12 were more effective inducers than types 6 and 16. The multiplicity of infection was approximately 1000–100,000-fold higher in the cells infected with types 6 and 16; yet, less interferon was induced than in cells inoculated with types 8 and 12.

Interferon induction by types 6, 8, 12 and 16 in chicken leukocytes. Differences among human types regarding their ability to induce interferon could also be demonstrated in chicken leukocytes. The titres of interferon produced in type 8 and 12 infected leukocytes were considerable higher than those obtained from type 6 or 16 infected cells (Table II).

Electron microscopy of HEp-2 cells infected with types 6, 8, 12 and 16. Adenoviruses were inoculated into monolayer cultures of HEp-2 cells. The multiplicities of infection were 10, 0.1, 0.01 and 0.00001 in cells infected with

Table I*Titres of interferon induced by types 6, 8, 12 and 16 in chick embryo fibroblast cells*

Virus		Titre of interferon
Type	Dose (log ₁₀ TCID ₅₀)	
6	7.5	64
	6.5	4
	5.5	<4
8	1.5	128
	0.5	32
12	4.5	1024
	3.5	64
16	5.75	32
	4.75	<4

Table II*Titres of interferon induced by types 6, 8, 12 and 16 in chicken leukocytes*

Virus		Titre of interferon
Type	Dose (log ₁₀ TCID ₅₀)	
6	8.8	64
	7.8	4
8	1.8	256
	0.8	32
12	4.8	2048
	3.8	256
16	5.8	64
	4.8	< 4

types 6, 16, 12 and 8, respectively. Samples for electron microscopic examination were taken at daily intervals for 4 days. Cells infected with any type revealed intracellular progeny virus about 16 hr after infection. Pronounced changes in cell morphology occurred 72 hr after infection when the nuclei of most cells contained virus particles.

Figure 1 illustrates the nucleus of an uninfected HEp-2 cell. Non-viral paracrystals were found in cells infected with type 6. The paracrystalline structures were first detected at 72 hr in the nuclei usually among virus particles. No virus particles were found within the crystalline lattice (Figs 2 and 3). The fine structure of the paracrystal consists of regularly repeated filaments (Fig. 4). The space between two filaments is about 40 nm.

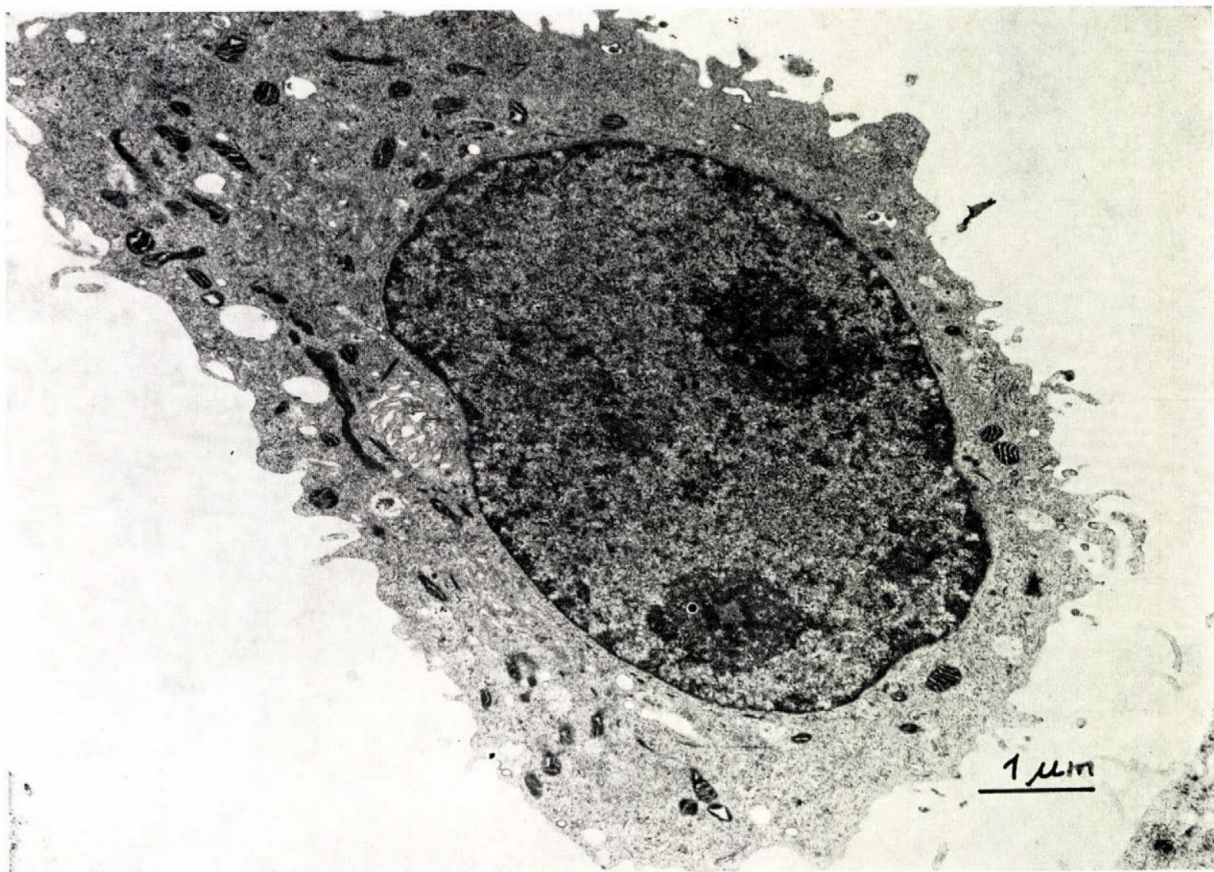


Fig. 1. Electron micrograph of a noninfected HEP-2 cell. Two nucleoli are visible in the nucleus. $\times 15\,000$

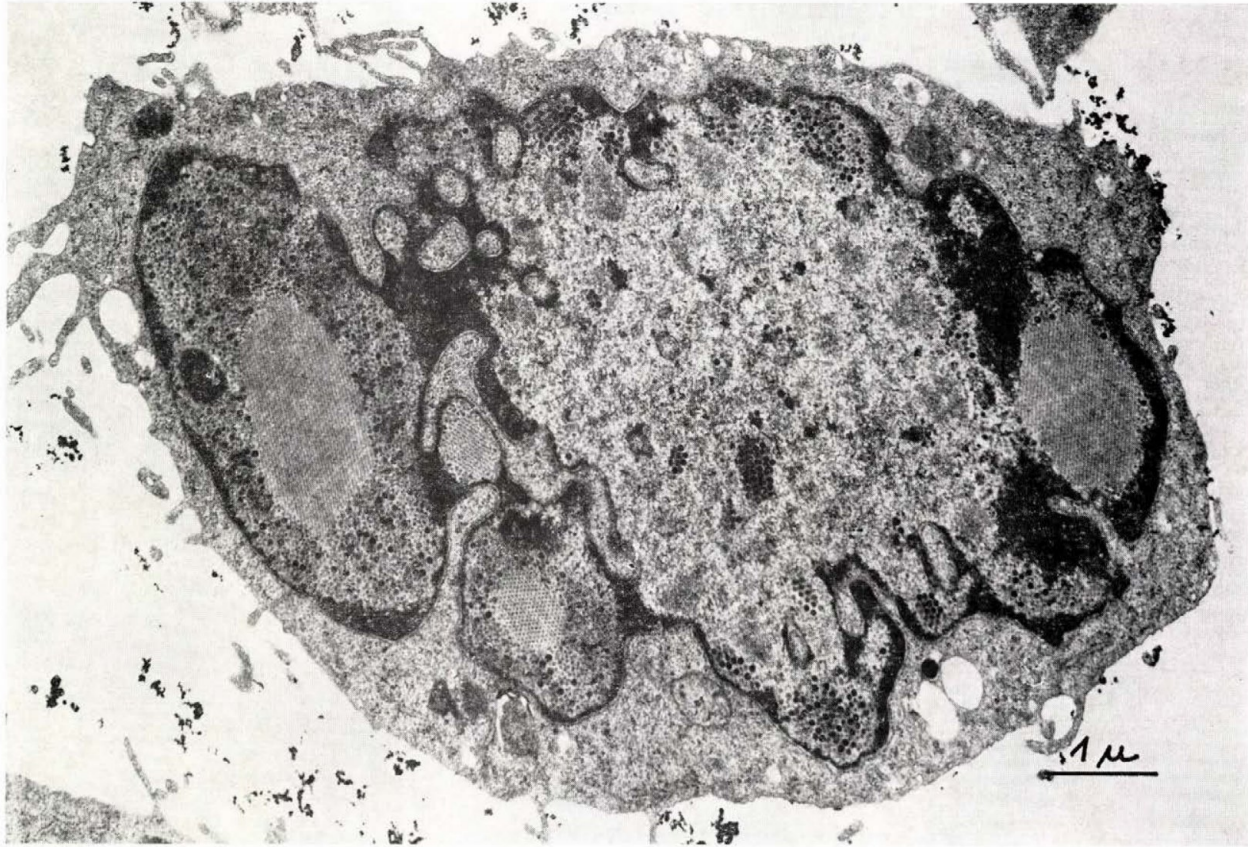


Fig. 2. Electron micrograph of a HEp-2 cell 72 hr after infection with type 6. Two large intranuclear paracrystals are present. $\times 25\,000$

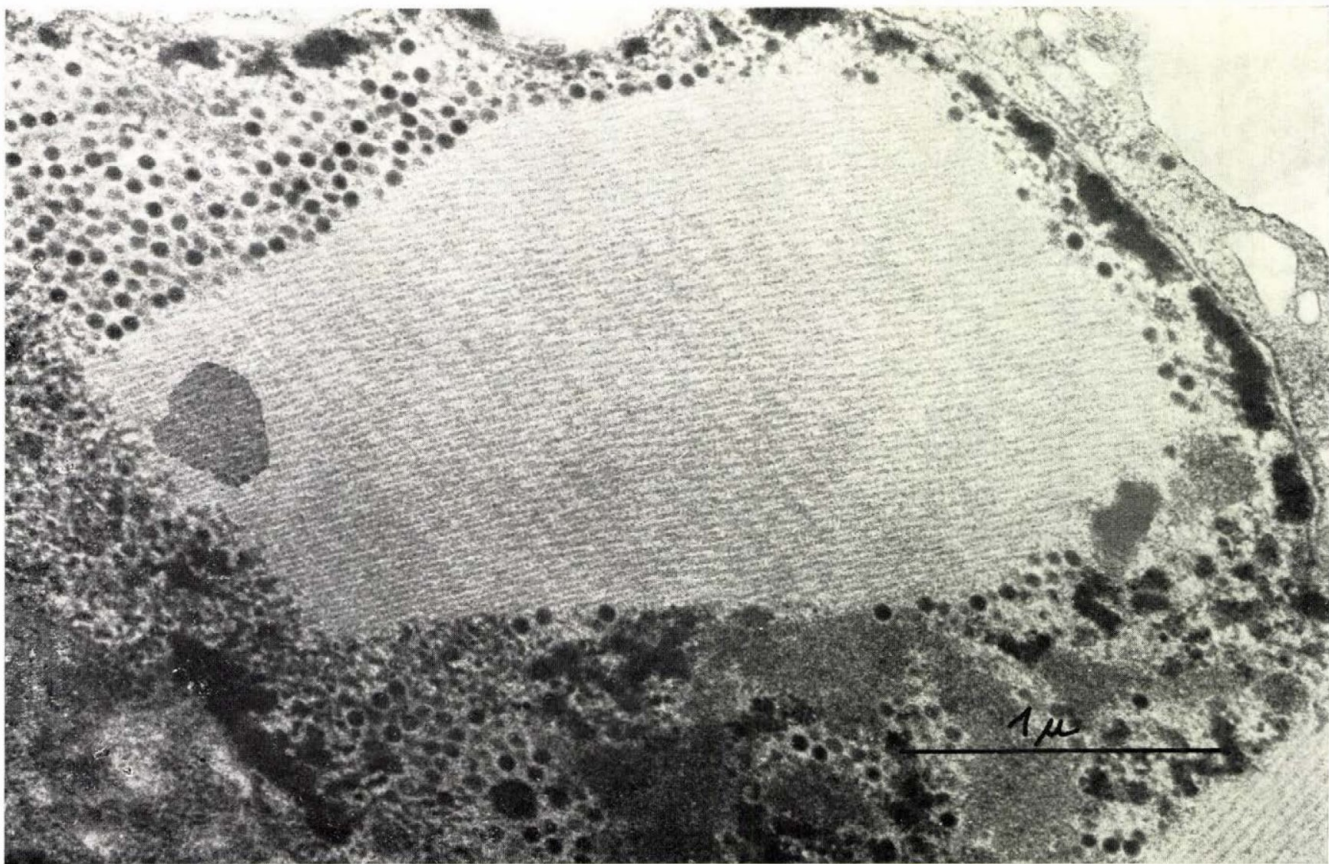


Fig. 3. Portion of a nucleus of a HEP-2 cell 72 hr after infection with type 6. The paracrystalline structure is surrounded by virus particles. $\times 42500$

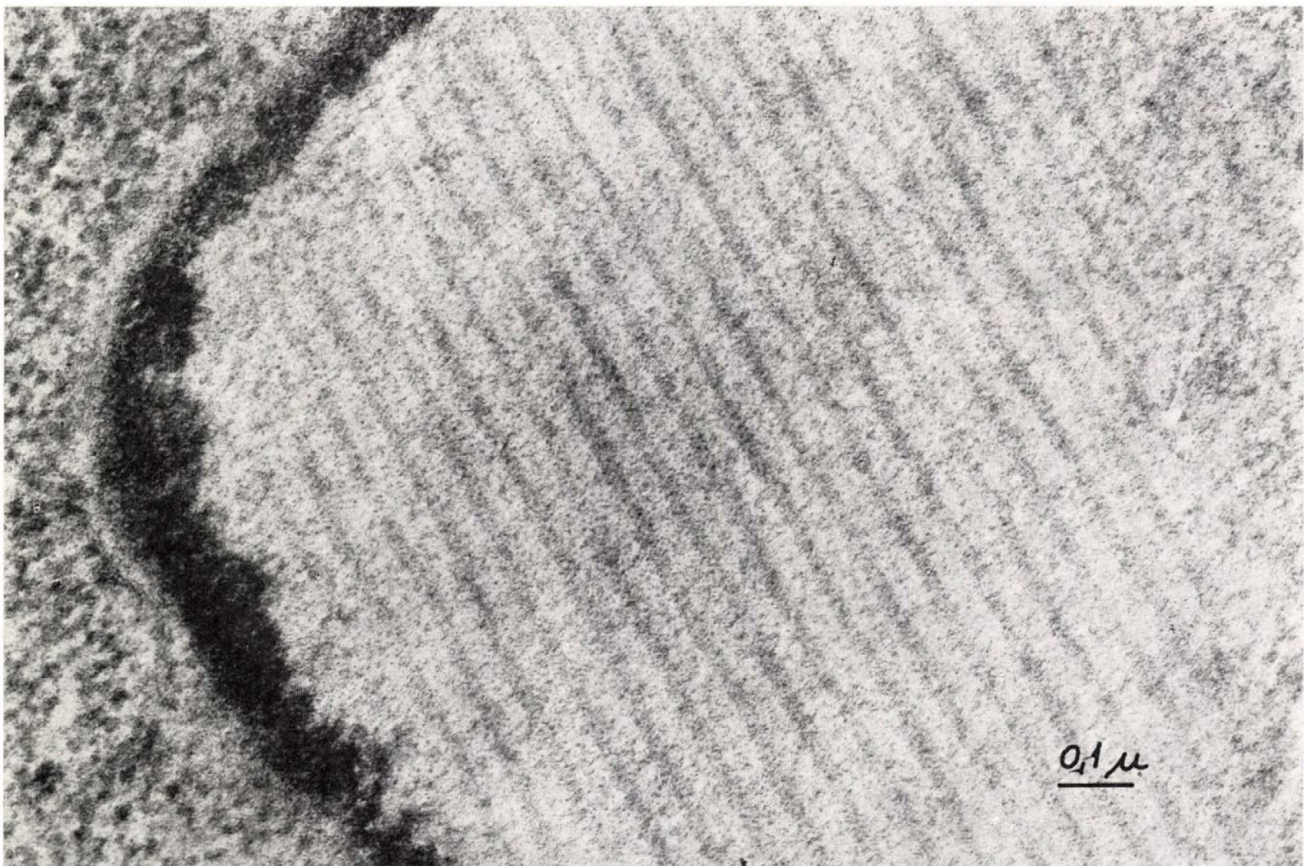


Fig. 4. Higher magnification of a paracrystal in type 6 infected cell, $\times 100\,000$

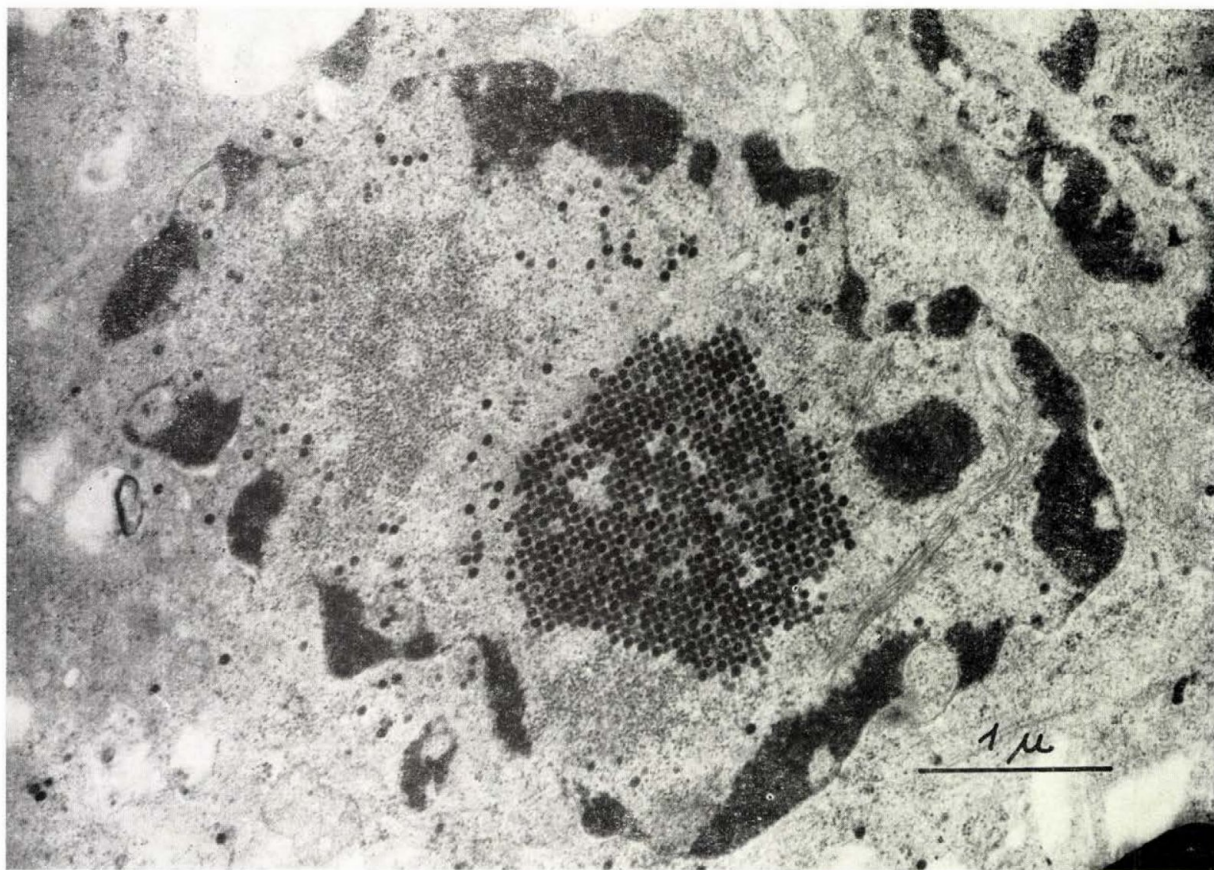


Fig. 5. Electron micrograph of a HEP-2 cell 72 hr after infection with type 8. Large viral crystal in the nucleus. $\times 25\,000$

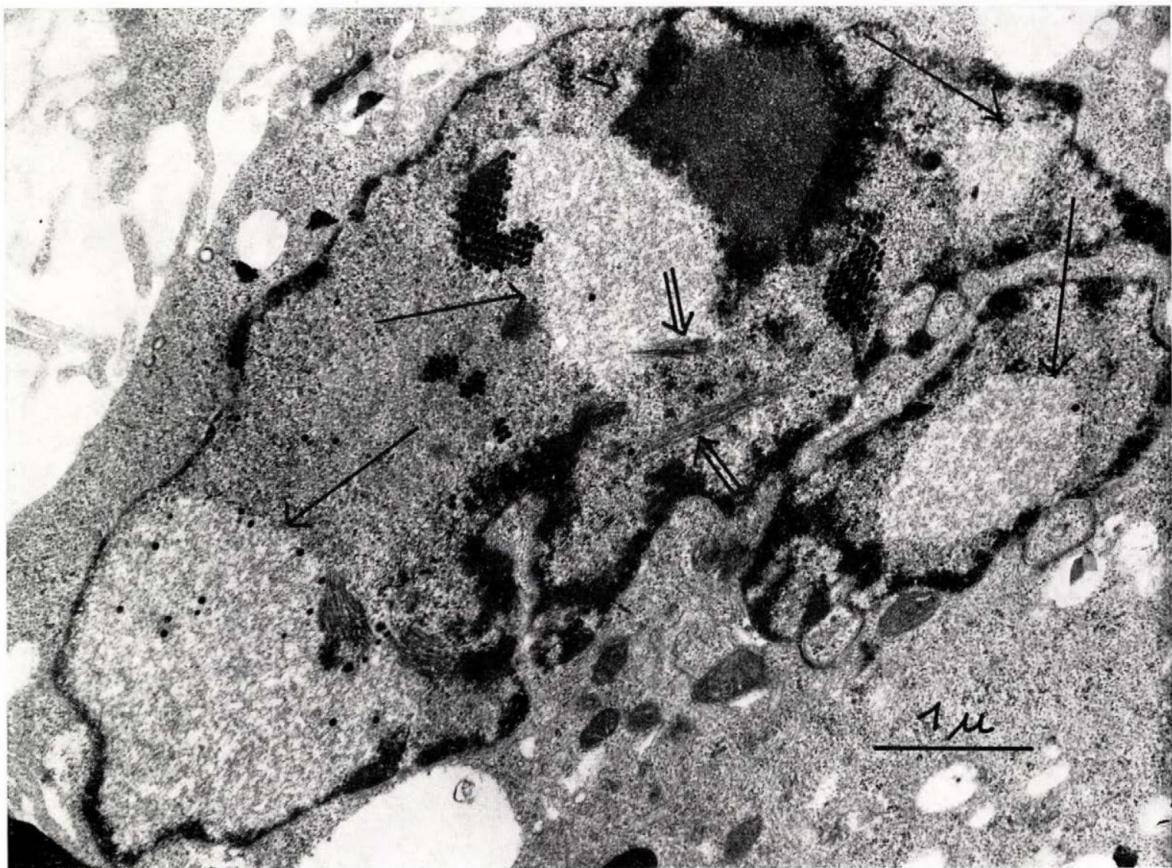


Fig. 6. Inclusions type II (small arrow), type III (large arrows) and fibre bundles (double arrows) in a HEp-2 cell 72 hr after infection with type 8. $\times 20\ 000$

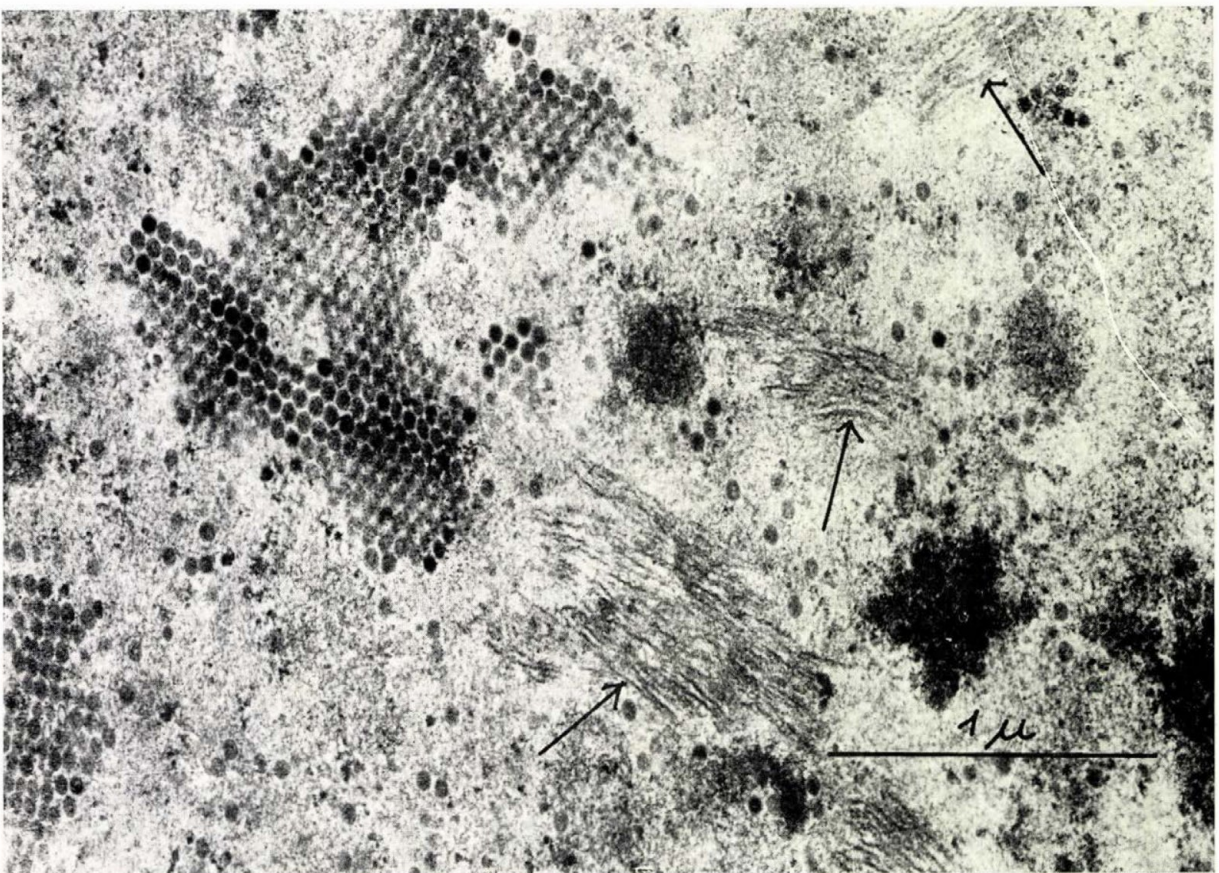


Fig. 7. Portion of a nucleus of a type 8 infected cell. Bundles of fibres (arrows) are seen beside the viral particles in crystalline arrangement. $\times 42\,500$

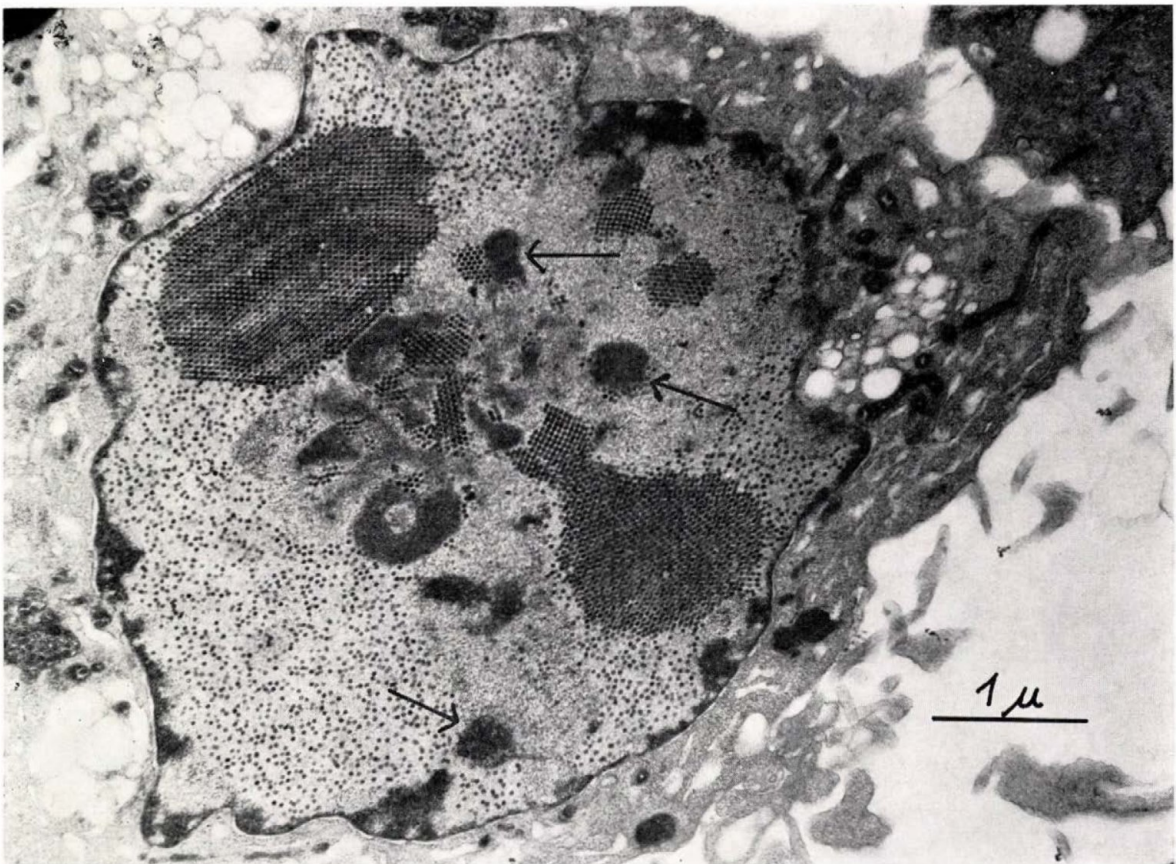


Fig. 8. Electron micrograph of a HEp-2 cell 72 hr after infection with type 12. Viral crystalline arrays and scattered viral particles in the nucleus. Electron-dense type I inclusions (arrows). $\times 20\,000$

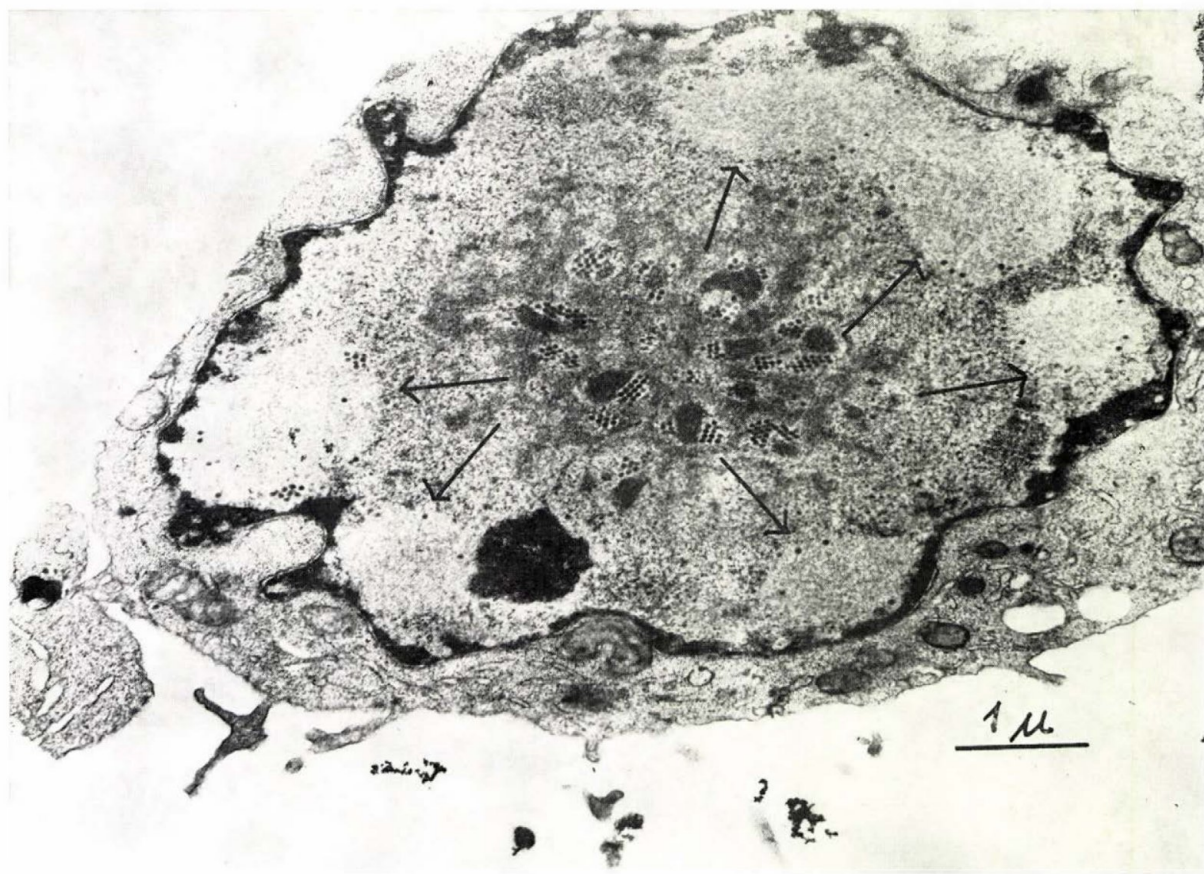


Fig. 9. Electron micrograph of type 12 infected HEp-2 cell (72 hr postinfection). The nucleus contains several type III inclusions at the nuclear membrane (arrows). $\times 17\ 000$

In HEp-2 cells infected with type 8, the virus particles could be observed in a crystalline array (Fig. 5). Intranuclear inclusions were also found (Fig. 6) which were similar to types II and III inclusions described by MARTINEZ-PALOMO *et al.* [11]. The appearance of fibre bundles could be detected in most nuclei. Figure 7 illustrates the fibres at higher magnification in the vicinity of virus particles in crystalline arrangement.

In cells infected with type 12 progeny virus particles in crystalline arrangement and scattered throughout the nucleus were observed. Type I inclusions in moderate number were also present (Fig. 8). A large number of inclusions similar to type III inclusions were found (Fig. 9).

After 48 hr incubation, the nuclei of HEp-2 cells infected with type 16 contained large numbers of virus particles, many of them forming virus crystals (Fig. 10). Both the nucleus and the cytoplasm contained virus particles at 72 hr incubation (Fig. 11).

No paracrystal formation was observed in HEp-2 cells infected with either types 8, 12 or type 16.

Discussion

Previous studies have demonstrated the presence of paracrystalline structures in cells infected with different adenoviruses [12-15]. It has also been found that not all strains of adenoviruses stimulated paracrystalline formation. The significance of non-viral crystal formation in virus infected cells has not been elucidated. BOULANGER *et al.* [16] assumed a relationship between the paracrystals induced and the capsid components. In contrast, MARUSYK *et al.* [17] demonstrated that antiserum against the core protein of adenovirus reacted with the paracrystals. Recently, WILLS *et al.* [18] have found that paracrystals are related to the fibre antigen of the virus. Even though the paracrystals have been found to be related to the capsid component of the virus, their exact role in the cells in which adenovirus replicates is not known.

Crude adenovirus material obtained from the infected cells by 5 times freezing and thawing has been used for interferon induction. Therefore, non-viral crystals could be present in our virus materials. The possible role in interferon induction of the strain-dependent paracrystalline formation was supported by the fact that different human types exhibited a different interferon inducing ability, further the finding that trypsin-treatment destroyed or markedly reduced the capacity of inducing interferon formation by human adenoviruses in chick embryo fibroblast cells [5].

In this study, different morphological alterations could be observed in HEp-2 cells infected with types 6, 8, 12 and 16. Type III inclusions were demonstrated in cells infected with types 8 and 12. However, paracrystalline

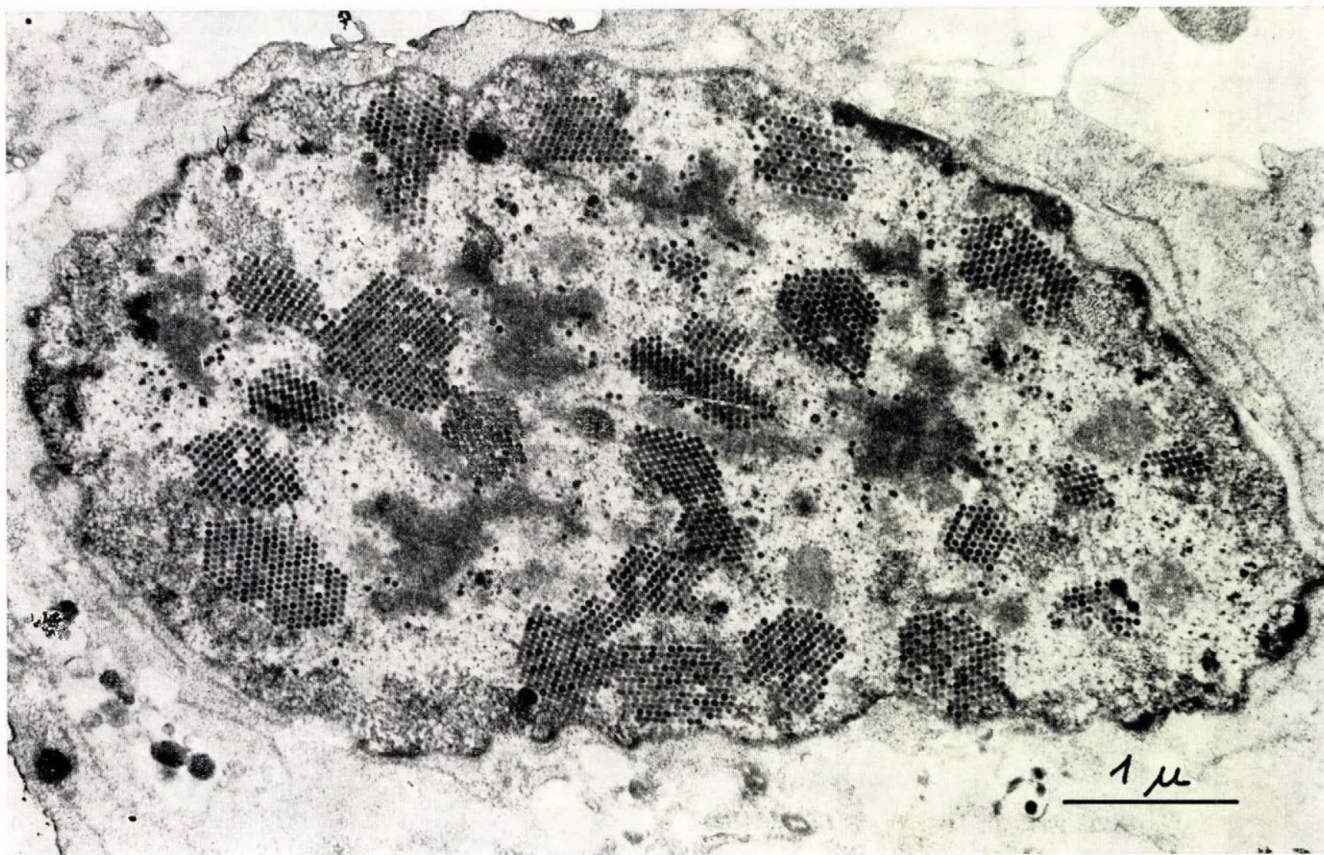


Fig. 10. Electron micrograph of HEP-2 cell 48 hrs after infection with type 16. A large number of viral crystals is seen in the nucleus. $\times 22\,500$

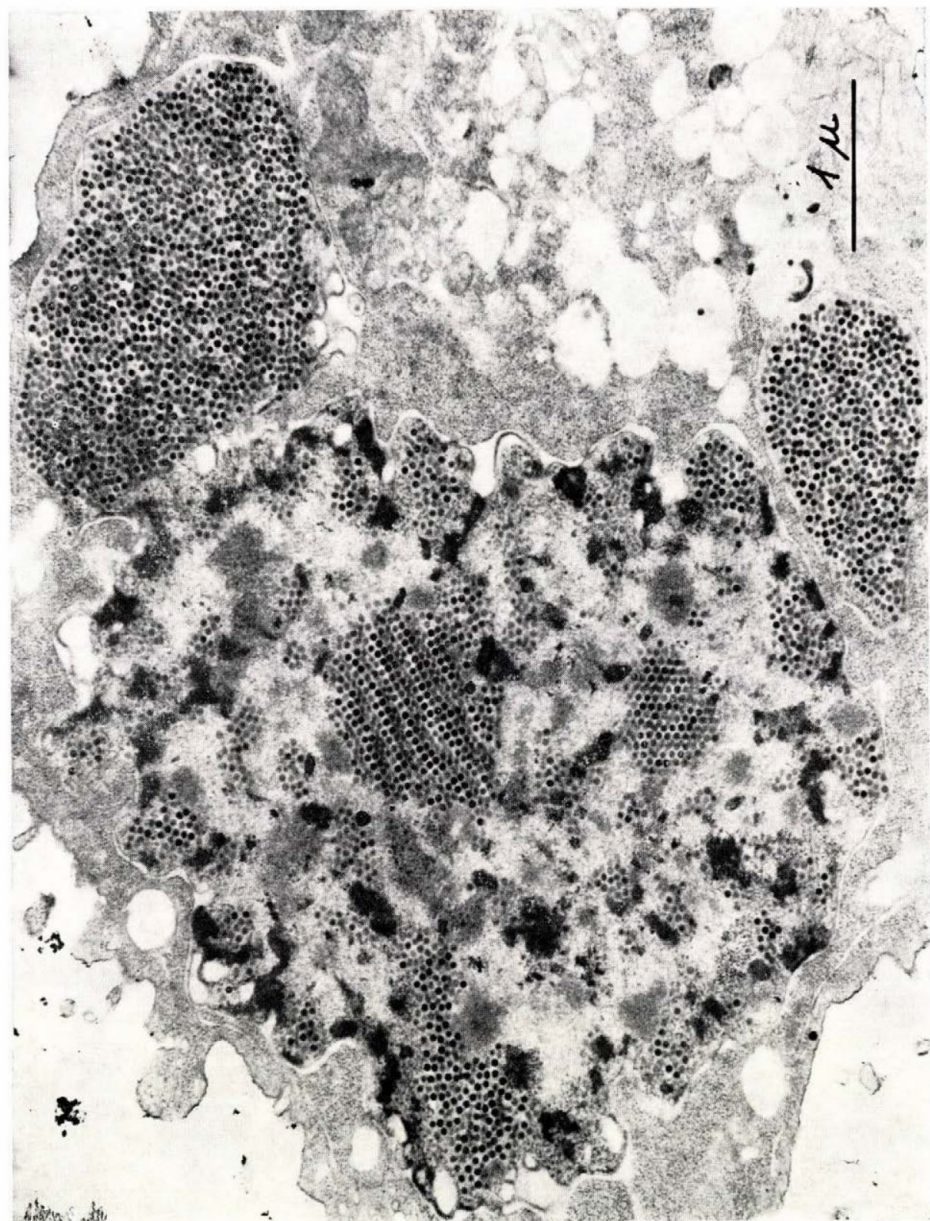


Fig. 11. HEp-2 cell 72 hrs after inoculation with type 16. Large aggregates of viral particles in the nucleus and in the cytoplasm are evident. $\times 22\,500$

formation was only found in type 6 infected cells. The effective interferon inducers, *i.e.* types 8 and 12, did not stimulate the formation of non-viral crystals in HEp-2 cells and this was also absent in type 16 infected cells. These evidences suggested that paracrystalline formation was independent of the ability to induce interferon by a certain human adenovirus strain. The possible role of type III inclusions in interferon induction remains to be elucidated.

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BACTERIOLOGICAL AND PHARMACOLOGICAL INVESTIGATIONS WITH GARAMYCIN® (GENTAMICIN)*

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(Received December 13, 1973)

Summary. Garamycin (gentamicin) sensitivity of bacterial strains isolated in hospital environment and concentration of the drug in serum, lung, kidney and urine were determined. The incidence of sensitive strains was: *Staphylococcus aureus* 95, *Pseudomonas aeruginosa* 94, *Acinetobacter* 93, *Proteus-Providencia* 97, other *Enterobacteriaceae* 99–100, *Haemophilus influenzae* 90%. Two hours after intramuscular injection of 240 mg Garamycin the organs showed a sufficient therapeutic concentration of the drug.

Gentamicin, an aminoglycoside antibiotic has a wide range of activity against a number of Gram-negative and Gram-positive microorganisms. This antibiotic has proved to be of special value in various severe infections due to bacteria with multiple resistance [1–3].

The purpose of our investigations was to study the effectiveness of Garamycin (Schering Co., U. S. A.) *in vitro* against bacterial strains occurring in our hospital area and to determine the drug concentration in human tissues.

Materials and methods

In vitro studies. The antibacterial spectrum of Garamycin was studied by standardized disc sensitivity method described by BAUER *et al.* [4], with 10 µg drug/disc as tentative standard. The antibiograms were determined on R-agar plates (Human, Budapest). Five per cent defibrinated sheep blood were added to the agar base to improve the growth of more fastidious organisms such as streptococci and haemophilus. Incubation lasted for 20 hr at 37°C. Bacteria were scored as sensitive to the drug in the zones of inhibition measured 15 mm or more in diameter; for *Pseudomonas aeruginosa* 12 mm or larger zones were considered as a criterion of sensitivity [5].

Determination of antibiotic level in tissues. Soon after the opus the kidney and lung were washed with sterile saline, decapsulated and thoroughly dried with sterile gauze. Then minced to pieces about lumps of sugar in size and homogenized in a Biomix homogenizator (Labor MIM, Budapest) for 5 min at 18,000 rpm. Complete destruction of cells was checked microscopically. To 10 g of tissue homogenizate 10 g of phosphate buffer (pH 8) was added. From the stock suspension dilutions 1 : 3, 1 : 4, 1 : 5 and 1 : 6 were made. Agar wells on 5 parallel test plates were filled with the tissue homogenizates and incubated for 18 hr at 37°C.

Test agar plates. A *Staphylococcus aureus* ATCC-6538P overnight nutrient agar slant culture was harvested with buffer (pH 8) and standardized to BaSO₄ suspension (0.5 ml 1% BaCl₂ + 99.5 ml 1% concentrated sulfuric acid) by photonephelometry. Then 2 ml of bacterial suspension were added to 98 ml melted 50–52°C agar, adjusted to pH 8 (Grove-Randall medium). Fifty ml of the inoculated agar were poured into big Petri dishes (150 mm in diam-

* This study is part of the paper given at the VIIIth International Congress of Chemotherapy held in Athens, Kriti, Rhodes and Cos, 8–15 September, 1973.

eter). These plates could be used for 5–6 days when stored at 4°C. Agar wells were made with a cork-drill 8 mm in diameter.

Garamycin standard. Garamycin base solution (100 µg/ml) was made in pH 8 phosphate buffer. From this, dilutions were performed 10, 8, 6, 4, 2 and 1 µg/ml in the same buffer solution. On every test agar plate all standard dilutions from 1 to 10 µg/ml were tested.

Assay. The inhibition zone diameters of standard dilutions were measured and the parallels averaged. Then plotted on semilogarithmic paper versus concentrations using the abscissa for diameters in mm. From the obtained diagram the final result of averaged tissue inhibition zones were extrapolated and multiplied by the dilution of the original specimen.

The concentration of the drug in the body fluids (serum, urine) was determined by the same agar diffusion method using the patients' own serum or urine as diluent taken before the administration of Garamycin. Urine samples containing the drug were adjusted to pH 8 and diluted in patients own urine (pH 8) without drug.

Results

Antibacterial effect of Garamycin. A large number of various bacterial strains were tested by disc method against Garamycin. From the total of 1656 strains 1396 were Gram-negative rods such as *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus Providencia*, *Citrobacter*, *Salmonella*, *Shigella*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Acinetobacter anitratus* and *hwoffii*, *Alcaligenes*; 260 penicillin resistant *Staphylococcus aureus* strains were studied in addition. The results are shown in Table I.

Table I
Garamycin sensitivity of isolates

Organism	No. of strains	Sensitive	Resistant
<i>Escherichia</i>	626	621 (99%)	5 (1%)
<i>Klebsiella</i>	121	121 (100%)	—
<i>Enterobacter</i>	171	169 (99%)	2 (1%)
<i>Serratia</i>	5	5 (100%)	—
<i>Proteus-Providencia</i>	215	208 (97%)	7 (3%)
<i>Citrobacter</i>	13	13 (100%)	—
<i>Salmonella</i>	6	6 (100%)	—
<i>Shigella</i>	4	4 (100%)	—
<i>P. aeruginosa</i>	118	111 (94%)	7 (6%)
<i>H. influenzae</i>	75	67 (90%)	8 (10%)
<i>Acinetobacter</i>	32	30 (93%)	2 (7%)
<i>Alcaligenes</i>	10	9 (90%)	1 (10%)
<i>S. aureus</i> (penicillin resistant)	260	247 (95%)	13 (5%)
Total	1656	1611 (97.3%)	45 (2.7%)

Garamycin in tissues. Tissue levels of Garamycin were determined in the kidney from 5 nephrectomized patients suffering from end stage renal disease, and in the lung of five patients who were lobectomized or resected after the administration of the drug. All patients received 240 mg Garamycin i.m. two hr before operation.

In the above organs a sufficient therapeutic concentration was reached. Fig. 1 shows the average levels of Garamycin in serum, lung, kidney and urine.

Despite the high serum levels and extremely high urine concentrations the drug showed a lower level in the tissues. This finding can be explained by the fact that infected or tumour tissues were examined.

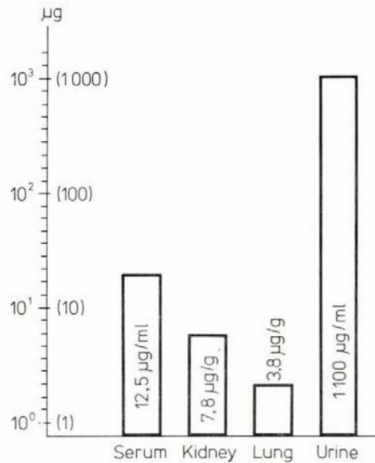


Fig. 1. Garamycin concentrations 2 hr after administration (240 mg i.m.)

Discussion

Our results on the sensitivity *in vitro* of bacteria isolated in our hospital environment agree with other authors' studies [6, 7]. The present studies have confirmed that the drug reaches a sufficient therapeutic concentration in human tissues, namely in kidneys, lungs and blood and an extreme high level in urine.

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COMPARISON OF *ALCALIGENES FAECALIS* AND *ALCALIGENES ODORANS* VAR. *VIRIDANS* BY CARBON SOURCE UTILIZATION TEST

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Summary. Thirteen strains of *Alcaligenes faecalis* and 10 strains of *Alcaligenes odorans* var. *viridans* were compared with the carbon source utilization test involving 64 organic substances. Of these, 24 supported growth of the strains of both species, 31 compounds were not utilized, the utilization data of 9 compounds were not uniform. The utilization spectra of the investigated *A. faecalis* and *A. odorans* var. *viridans* strains showed a close taxonomic relationship of the two species.

According to present knowledge, *Alcaligenes faecalis* and *Alcaligenes odorans* are bacteria belonging to the genus *Alcaligenes*. Organisms belonging to these species show a close phenotypic similarity and only few characteristics of differential value. The most remarkable feature is the capacity of *A. odorans* cultures to produce an aromatic odour. The major part of the strains belonging to the latter species produces haemolysis and a green colour on blood agar, therefore they are named as *A. odorans* var. *viridans* [1].

Carbon source utilization tests have been used recently in a number of extensive taxonomic studies. The ability of bacteria to utilize organic carbon compounds as sole carbon and energy source may be a characteristic of taxonomic value. The purpose of the present work was to study whether there are any characteristics distinguishing *A. faecalis* and *A. odorans* var. *viridans* with the aid of the carbon source utilization test.

Materials and methods

Organisms. Thirteen strains of *A. faecalis* and 10 strains of *A. odorans* var. *viridans* were involved in the investigation. Most organisms were isolated from various human materials, some strains were obtained from the National Collection of Type Cultures (London) and from the American Type Culture Collection (Rockville, Maryland) by the courtesy of Dr. S. P. LAPAGE and Dr. E. F. LESSEL. The list of the collection strains is given as follows: *A. faecalis* NCTC 415, NCTC 655, NCTC 8764, ATCC 19018, ATCC 213, ATCC 8748, ATCC 8750 and *A. odorans* var. *viridans* NCTC 10388.

The main characteristics of the strains were controlled repeatedly. Each organism was motile, peritrichously flagellated; they gave a positive oxidase reaction, grew aerobically and produced an alkaline reaction in the O-F glucose medium of Hugh and Leifson. The strains, except one, did not reduce nitrate, they did not produce lysine and ornithine decarboxylase, arginine dihydrolase and urease. All strains grew at 22°C and 37°C and some at 42°C. Gelatin liquefaction was not observed after three weeks incubation.

Carbon source utilization test. The basal mineral medium contained ammonium sulphate, 1 g; magnesium sulphate, 0.2 g; sodium chloride, 0.3 g; 0.04 M phosphate buffer (pH 7.0)

1000 ml. A mixed solution of metal salts was added to the medium to a concentration of 0.02–10.0 mg per litre for the various cations, according to STAINER *et al.* [2]. The organic substances were incorporated into the medium at a concentration of 0.2%, except the DL-amino acids, the concentration of which was 0.4%. Sugars were added to the medium in sterile filtered solution, other materials were sterilized after mixing to the medium at 110°C. Before sterilization, the media were distributed into tubes in 3 ml amounts.

The bacterial strains to be tested were inoculated onto agar slopes and the cultures grown were washed off with 10 ml of saline after 24 hr incubation at 37°C. One drop (0.05 ml) of this suspension was used as inoculum. Results were read on the 2nd, 4th and 6th day of cultivation.

Results

Results are summarized in Table I. According to their ability of supporting growth of *Alcaligenes* strains, the 64 substrates could be ordered in three groups. The members of the first group served as carbon and energy source equally for *A. faecalis* and *A. odorans* var. *viridans*. Some amino acids, alcohols and the substances of the oxidation pathway of the cell belonged to this group. One of the *A. faecalis* strains (NCTC 8764), which did not utilize the substances ordered in the 2nd, 3rd and 4th line of the Table I, differed also in colony morphology and nitrate reduction from typical strains of *A. faecalis*. At the bottom of Table I are found the substances which failed to support growth of *A. faecalis* and *A. odorans* var. *viridans*. Amino acids and carbohydrates constituted the major part of this group. One strain of *A. faecalis* could utilize glucose, fructose and cellobiose in repeated experiments after careful control

Table I
Utilization of organic compounds by *Alcaligenes* strains

Compounds tested	<i>A. faecalis</i> 13 strains	<i>A. odorans</i> 10 strains
DL- α -alanine, DL-aspartate, L-gutamate, DL-phenylalanine, L-proline, ethanol, propanol, isobutanol, acetate, succinate, lactate, citrate, malonate, pyruvate, D-malate, α -keto- glutarate, acetamide	13*	10
L-glutamine, citraconate	12	10
glycine, L-leucine, DL-norleucine, DL-isoleucine, DL-methionine	11	10
L-ornithine	11	5
maleate, nicotinate, nicotinamide	8	10
DL-tryptophane	8	9
L-histidine	3	10
L-lysine	6	—
glycerol	2	—
DL-valine	1	6
p-hydroxybenzoate	1	1
DL-asparagine, β -alanine, kynurenate, glucose, fructose, cellobiose, folic acid, adipate, 2,3-butanediol	1	—
DL-serine, L-arginine, DL-threonine, citrullin, xylose, lactose, sucrose, raffinose, mannitol, dulcitol, m-inositol, adonitol, methanol, isovalerate, mesotartrate, oxalate, gluconate, aniline, ethyleneglycol	—	—

* Number of utilizing strains

of identity and purity of culture. A small part of the substances belonged to the third group. In the case of these compounds, the utilization spectra of *A. faecalis* and *A. odorans* var. *viridans* were different. The differences between the two species could be reproduced with some variations, but there was no compound in respect of which the difference would have proved significant.

Discussion

Data on the similarity of *A. faecalis* and *A. odorans* var. *viridans* are scarce in the literature. DE LEY *et al.* [3] have shown that the strains of these two species are related very closely, since their per cent guanidine-cytosine values are in a narrow zone between 56 and 59 and the strains of both species failed to show any activity of the enzymes D-gluconate-6-phosphate dehydratase and 2-keto-3-deoxy-D-gluconate-6-phosphate aldolase. In the course of a previous survey we found the overall similarity of *A. faecalis* and *A. odorans* var. *viridans* to be very close, and using morphological and conventional bacteriological tests we could not separate the two species from each other by numerical methods [4].

On the basis of the utilization pattern of 64 various organic compounds, no sharp distinction could be made between the two species. This observation is indicative of a close taxonomic relationship. According to present knowledge, the most important differences between the species are the colony morphology on blood agar and the capacity of *A. odorans* var. *viridans* to produce nitrogen gas from nitrite [5].

RHODES [6] described a soil bacterium capable of utilizing aniline and she proposed to order this organism into the genus *Alcaligenes*. Though the organism is different from *Alcaligenes* strains in respect of some other characteristics, too, our strains belonging to both of these species did not utilize aniline.

The present data showed a high biochemical similarity of *A. faecalis* and *A. odorans* var. *viridans*. This close relationship does not, however, contradict their classification into two separate species.

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STUDY OF AUXOTROPH MUTANTS INDUCED BY COPPER SULPHATE IN *BACILLUS SUBTILIS*

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Summary. Auxotroph mutants were isolated from 168 *Ind*⁻ streptomycin sensitive (*Sm S*) and 168 *Ind*⁻ streptomycin resistant (*Sm R*) strains of *Bacillus subtilis* after CuSO₄ treatment. Auxotrophs isolated from the *Sm S* strain could be classified into two groups. (1) Isolates with determinable auxotrophies; after adding various amino acids, nucleic bases or vitamins, the growth rate of these mutants was similar to that of the parent strain. (2) "Minute" mutants. Their auxotrophy could not be determined. They grew very slowly, the maximum diameter of the colonies after four days incubation did not exceed 0.5 mm. They were resistant to the virulent phages SP50, SP51 and SP60 as well as to the temperate phages PBS-1 and 3NT. They could not be transformed with the DNA isolated from the parent strain. Reversion to the wild type was frequent. From the *Sm R* strain, haemin-requiring auxotrophs were isolated in addition to the former two groups.

The effect of CuSO₄ on bacteria has been studied by several authors. WEED and LONGFELLOW [1] and HIRSCH [2] isolated various mutants from *Escherichia coli* populations exposed to the effect of copper. WEED [3] investigated the effect of copper sulphate on *Bacillus subtilis*. ANDERSON and IVÁNOVICS [4] isolated haemin-auxotrophs from *B. subtilis* following CuSO₄ treatment.

Our aim was to isolate new haemin-requiring auxotrophs. Beyond this, we have isolated other strains with definable auxotrophy and mutants the auxotrophy of which could not be determined. The latter slowly growing variants were tested for phage resistance, transformability, temperature sensitivity and streptomycin resistance.

Materials and methods

Strains. The *Sm R*, an isolate of high streptomycin-resistance tolerating even 100 µg/ml of streptomycin, and *Sm S*, an isolate showing the original low streptomycin-resistance of the parent strain, tolerating 15 µg/ml of the antibiotic were isolated from *B. subtilis* 168 *Ind*⁻ strain having the same tryptophan (or indole) auxotrophy.

Phages. SP50, SP51 and SP60 virulent phages were isolated and characterized by FÖLDES and MOLNÁR [5]. 3NT and PBS-1 temperate phages were described by CSISZÁR and IVÁNOVICS [6] and TAKAHASHI [7], respectively.

Media. GGM, a minimal medium containing glutamate and glycerol [4]. It was completed with 50 µg/ml tryptophan. YP, a complete medium containing yeast extract and peptone [6]. YPB, YP medium with 1% v/v human blood stabilized with sodium citrate. HAC, minimal medium containing haemin, albumin and cysteine [8]. The media were solidified with 1.5% w/v agar-agar.

Determination of minimum nutritional requirement. The isolates were grown first on YPB then transferred to YP and YPB agar. If the isolate seemed to be porphyrin dependent, it was inoculated onto GGM-ALA (5-aminolaevulinic acid, 2 $\mu\text{g/ml}$) and HAC media. If the isolate grew on YP agar, but not on GGM agar, its specific requirement was tested for all of the amino acids, nucleic bases and 11 water-soluble vitamins.

Determination of phage-resistance. In the case of the "minute" strains, a thick suspension of the bacteria was plated on YPB agar with a drop of the phage preparation. With all the other strains, the bacteria were mixed with 40°C agar medium and poured into Petri dishes. Drops of the phage preparations were placed onto the surface of the agar plates.

Isolation of DNA. Bacteria were harvested in the late exponential phase from aerated cultures in YP medium, washed in GGM medium and the DNA was prepared according to MARMUR [9]. DNA concentrations were determined using the method of BURTON [10].

Transformation experiments. The method of ANAGNOSTOPOULOS and SPIZIZEN [11] was followed with some modifications (see text).

Isolation of auxotrophs. *Sm R* and *Sm S* strains were cultured overnight on YP agar at 37°C. Bacteria were washed off with GGM, centrifuged and resuspended in GGM. Optical density was adjusted to 0.5 in a Baush and Lomb Spectronic 20 photometer at 620 nm. The suspension was divided into two parts. To the first part, CuSO_4 was added up to a 4×10^{-4} molar concentration while the other part served as control. The bacterial suspensions were incubated at 37°C without shaking. The number of colony formers was determined daily in both cultures. For isolating mutants, 0.1 ml samples were removed every second day from the CuSO_4 containing cultures, and plated on YPB agar. After 48 hr incubation, the colonies 0.5 mm or less in diameter were subcultured on YPB agar. After another 48 hr incubation, the subcultures were tested for auxotrophy. A typical experiment lasted 10 days.

The sensitivity to CuSO_4 of the strains was determined in GGM fluid medium under shaking. Sensitivity to temperature and streptomycin was tested on YPB agar.

Results

*Effect of CuSO_4 on *B. subtilis* 168 Ind⁻ in fluid cultures under shaking.* Growth of *Sm R* and *Sm S* strains was studied in GGM medium containing CuSO_4 at various concentrations (Figs 1 and 2). The *Sm S* strain seemed to be more sensitive than the *Sm R* above 2×10^{-4} mol CuSO_4 .

Isolation of auxotrophs. The change in the number of colony forming units during the experiment is shown in Fig. 3. The number of auxotrophs isolated from the cultures after different periods of time did not show any significant correlation to the time of exposition to CuSO_4 , so no distinction was made for the auxotrophs isolated after different periods of incubation.

The isolated mutants belonged to three classes (Table I), haemin auxotrophs were isolated only from the *Sm R* strain; 8 isolates of the 672 tested could be supplemented with haemin on minimal medium. Of eight, the auxotrophy of three could be supplemented with ALA, too. The majority of the other (non-haemin) auxotrophs turned out to have a requirement for amino acids. There were a few mutants with vitamin or nucleic base-auxotrophy. No significant difference was found in the frequency of these mutants between the *Sm S* and *Sm R* strains. The frequency of the "minute" strains was higher than 50% of all the mutants from both parent strains. They grew slowly and only on YP or YPB agar. The growth rate was better on YPB but the colonies did not exceed 0.5 mm in diameter after 96 hr incubation at 37°C. Attempts to determine their auxotrophic requirements have failed.

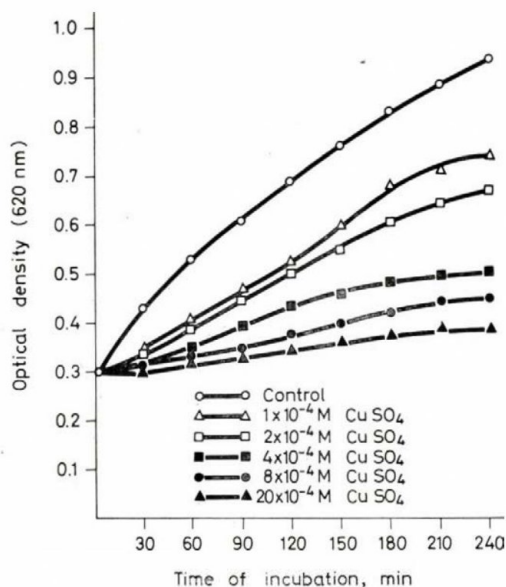


Fig. 1. Growth of *B. subtilis* 168 Ind- Sm R in GGM-tryptophan (50 µg/ml) fluid medium under shaking at different copper sulphate concentrations

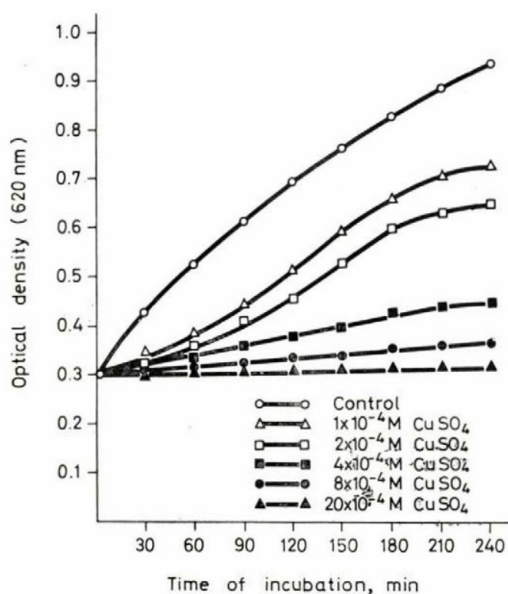


Fig. 2. Growth of *B. subtilis* 168 Ind- Sm S in GGM-tryptophan (50 µg/ml) fluid medium under shaking at different copper sulphate concentrations. CuSO_4 was added to the cultures at OD = 0.3. Increase of OD caused by the presence of CuSO_4 was corrected in the Figures. The number of colony forming units was determined at half hour periods for 4 hr

Table I
Distribution of the auxotrophs according to nutritional requirements

	Auxotrophy	No. of isolated mutants	
		Sm R*	Sm S**
Haemin auxotrophs	Haemin	5	0
	ALA	3	0
	"Minute"	54	41
Other auxotrophs	Proline	1	0
	Arginine	1	2
	Cysteine	2	1
	Histidine	4	3
	Threonine	2	0
	Leucine	1	2
	Isoleucine	2	1
	Valine	1	1
	Tyrosine	2	2
	Alanine	2	1
	Hydroxyproline	1	0
	Phenylalanine	1	1
	Histidine, cysteine	1	0
	Arginine, threonine	1	0
	Methionine, proline	1	0
	Methionine, cysteine	0	1
	Isoleucine, valine	0	1
	Aneurin (B ₁)	2	2
	Biotin	2	3
	Nicotinamide	1	1
	Adenine	2	3
	Guanine	1	2

* 93 auxotrophs were isolated from the 672 colonies tested

** 68 auxotrophs were isolated from the 1450 colonies tested

Characterization of "minute" strains. Phage-sensitivity. A 0.05 ml aliquot of a phage suspension containing 3×10^8 – 2×10^9 p.f.u./ml was dropped onto the bacteria plated on YPB agar. The result was evaluated after 48 hr incubation at 37°C. All the "minute" strains showed complete resistance to the SP50, SP51, SP60, PBS-1 and 3NT phages tested. Both the parent strains and all the haemin and other auxotrophs were sensitive to these phages.

Transformation. The strains were grown on YPB agar for 48 hr at 37°C. The bacteria were suspended in GGM, the optical density was adjusted to 0.4

and the culture was shaken in a 37°C water bath. After 1 hr, DNA isolated from the *Sm S* parent strain was added at 1, 5, 10 and 20 µg/ml final concentrations. The cultures were incubated at 37°C without shaking and samples taken at 60, 90, 120 min were plated on YPB agar. No transformant was found after 48 hr incubation at 37°C which would have shown the rapid growth rate characteristic of the wild type.

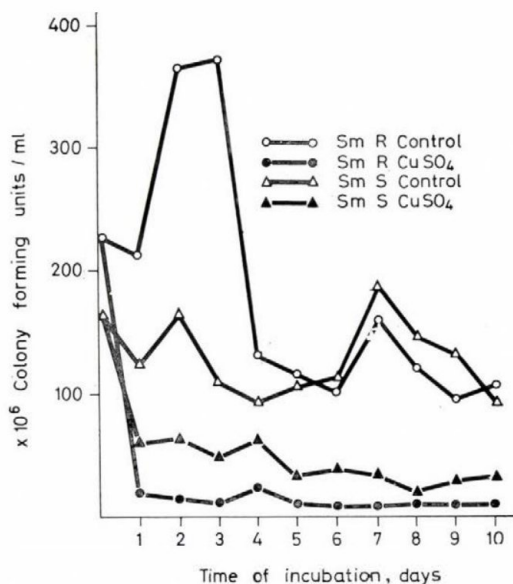


Fig. 3. Number of colony forming units during CuSO₄ treatment of strains *Sm R* and *Sm S*. Bacteria were incubated in 5 ml GGM fluid medium without shaking for 10 days at 37°C. The medium contained 50 µg/ml tryptophan and 4×10^{-4} M CuSO₄.

Temperature and streptomycin sensitivity. Ten strains, selected randomly from the "minute" derivatives of both the *Sm R* and *Sm S* parent strains, were tested. Table II shows the results after 72 hr incubation at 37°C. Derivatives of the *Sm S* strain remained sensitive to streptomycin and had even lost their low streptomycin resistance (5–10 µg/ml) characteristic of the parent strain [8]. Interestingly, derivatives of the *Sm R* strain had also lost the high streptomycin resistance of the parent strain (100 µg/ml). Heat resistance decreased significantly in the majority of the "minutes" examined.

Discussion

The mutants were isolated using the copper concentration method (4×10^{-4} M CuSO₄) of WEED [3]. According to our data, this concentration was the most suitable (Figs 1 and 2). Under shaking in GGM medium, the

Table II
Resistance to heat and streptomycin of the "minute" strains*

Strains examined	Diameter of the colonies (mm)**	5 µg/ml streptomycin	10 µg/ml streptomycin	20 µg/ml streptomycin	43°C	45°C
Derivates of <i>Sm R</i>						
M6	0.5	—	—	—	—	--
M7	0.5	—	—	—	—	—
M8	0.4	—	—	—	—	—
M9	0.5	—	—	—	—	—
M11	0.5	—	—	—	—	—
M12	0.3	+	—	—	+	—
M13	0.5	+	—	—	+	+
M14	0.3	—	—	—	+	—
M15	0.3	—	—	—	+	+
M16	0.5	+	—	—	+	+
<i>Sm R</i>	9.0	+	+	+	+	+
Derivates of <i>Sm S</i>						
M196	0.5	—	—	—	+	—
M211	0.5	—	—	—	+	—
M212	0.2	—	—	—	—	—
M213	0.2	—	—	—	+	+
M214	0.3	—	—	—	+	+
M269	0.2	—	—	—	+	—
M270	0.5	—	—	—	—	+
M272	0.4	—	—	—	--	+
M273	0.5	—	—	—	—	—
M298	0.4	—	—	—	—	—
<i>Sm S</i>	9.0	+	±	--	+	+

* The test was made on YPB solid medium at 37°C, incubation time was 72 hr

** Diameters were measured on isolated colonies located far from each other

Sm S parent strain was more sensitive to CuSO_4 than was the *Sm R* strain. Without shaking the opposite was true (Fig. 3).

The haemin auxotrophs could be isolated only from *Sm R*. MARJAI *et al.* [8] isolated haemin auxotrophs from the *Sm S* strain on a medium containing streptomycin at high (100–200 µg/ml) concentration. These isolates tolerated a higher concentration of streptomycin than did the parent strain, *i.e.* their streptomycin resistance was moderate. This could perhaps explain

why we have failed in isolating haemin auxotrophs from the *Sm S* strain by CuSO_4 treatment.

Strains with diverse but definable requirements belonged to the group of the other auxotrophs. When the required materials were added to the medium, these strains regained the original growth rate of the parent strain.

Isolation of "minute" variants has been described for several microorganisms. Variants of this type in yeast cultures observed by LINDEGREN *et al.* [12] and NAGAI *et al.* [13] proved to be respiration-deficient (RD) mutants. From *E. coli* cultures, small ("dwarf") colonies were isolated and characterized by COLWELL [14], WEED and LONGFELLOW [1], CLOWES and ROWLEY [15], NAGAI *et al.* [13] and HIRSCH [2]. Several essential characteristics such as cytochrome composition, lactose metabolism, UV-resistance, etc., of these isolates were altered in comparison to the parent strain. Such "dwarf" colonies were obtained from *Staphylococcus aureus* by YOUNG and DELVES [16], from *Salmonella* strains by STOKES and BAYNE [17], from "Micrococcus pyogenes", *Sarcina lutea* and *Shigella sonnei* by WEINBERG [18]. Similar types of mutants were described in the case of *B. subtilis* by WEED [3] as well as MARJAI *et al.* [8]. These "small-colony-mutants" could be induced by various factors such as antibiotics, metal ions, chemicals, ultraviolet irradiation, heat-treatment, etc., and had very diverse characteristics. Some of them were stable, segregating no revertants, others unstable. Their auxotrophy could be determined in several cases and could not in others.

Our "minute" strains were similar to those of MARJAI *et al.* [8] which had been isolated with streptomycin. They gave revertants rather frequently which made their manipulation difficult. They showed no auxotrophy. They had all lost their streptomycin resistance and their majority was heat-sensitive. As opposed to the parent strains, the "minute" isolates could neither be transformed nor transduced. These facts could be explained by assuming that the mutation responsible for the "minute" phenotype is a one-step mutation (frequent spontaneous reversion) and affects several characteristics of the cell pleiotropically at the same time. Their complete resistance to phages could be explained by a loss of the surface receptors indicating extensive changes in the cell wall and/or the cell membrane.

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FURTHER EVIDENCES THAT PRIMING IS A NON-ANTIVIRAL FUNCTION OF INTERFERON

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Summary. Pretreatment of L cells with cycloheximide completely inhibited their subsequent interferon production induced by polyinosinic-polycytidylic acid. Under similar experimental conditions, interferon production took place in cultures pretreated with cycloheximide in the presence of interferon. At the concentration employed, cycloheximide inhibited the antiviral effect of interferon. Except for a fall in the first 14 hr period, the degree of antiviral resistance of interferon-treated cells did not decrease considerably during 72 hr incubation in interferon-free medium. Conversely, the primed state of cells rapidly declined becoming undetectable after 24 hr incubation.

In addition to its well-known antiviral activity, interferon has been shown to exert non-antiviral effects on cells; for instance, the inhibition of tumour [1] and normal mammalian cells in culture [2] and the enhancement of specialized cellular functions [3–5]. The priming effect can also be included in this group if the term “antiviral” is interpreted at the level of intracellular events and not at the level of the organisms host defences. “Priming” denotes the phenomenon that interferon-treated cells produce earlier and usually more interferon and also have a higher susceptibility to interferon inducers than have untreated controls. The priming effect of interferon was described by ISAACS and BURKE [6] and their results have been extended and confirmed by several authors [7–15]. The mechanism by which interferon exerts its priming effect and the biochemical basis of the primed state of cells has not been clarified. STEWART *et al.* [14] suggested that the antiviral and priming activities of interferon were due to different mechanisms. In the present study we have attempted to separate the antiviral and priming effects and tried to find differences between the primed state and the antiviral resistance of cells by comparing their duration after interferon treatment.

Materials and methods

Materials. Cycloheximide was obtained from Fluka A. G. Buchs, Switzerland. DEAE-dextran (mol wt $\approx 2 \times 10^6$) was purchased from Pharmacia Fine Chemicals, Sweden. Polyinosinic acid (poly I) and polycytidylic acid (poly C) were obtained from P. L. Biochemicals Inc., U. S. A.

Tissue cultures. L cells were kindly provided by Dr. G. L. TOMS (University of Birmingham) and were grown in a medium consisting of Eagle's minimal essential medium with 10%

calf serum (CS) and antibiotics. The stationary L tube cultures (10^5 cells/tube) were seeded in one ml of medium and the administration of drugs was carried out in the same volume. If CS was omitted from the medium, it is stated below.

Virus. Vesicular Stomatitis Virus (VSV) was the same as used previously [16]. Preparation of poly I-poly C complexes from the synthetic single stranded polyribonucleotides, interferon production, assay of VSV and interferon were carried out as described earlier [12].

Testing of the effect of cycloheximide on the development of antiviral and priming activity of interferon. Stationary tube cultures of L cells were incubated at 37°C for 2 hr with cycloheximide ($25\text{ }\mu\text{g/ml}$) then for a further 22 hr with interferon (5 units/ml) in the presence of $10\text{ }\mu\text{g/ml}$ cycloheximide. Cultures, along with appropriate control cultures, were then washed three times with CS-free medium. After this procedure, groups of four tubes were selected and either infected with VSV (10^3 plaque forming units (p.f.u. per tube) or treated with $50\text{ }\mu\text{g/ml}$ poly I-poly C in the presence of $100\text{ }\mu\text{g/ml}$ DEAE-dextran. After 1 hr incubation at 37°C the poly I-poly C treated cultures were thoroughly washed and refed with medium. The fluids were harvested for virus assay from the cultures inoculated with VSV after 48 hr incubation. The yield of interferon was determined in the supernatants of poly I-poly C treated cultures at 12 and 24 hr intervals postinduction.

Testing of duration antiviral resistance and primed state. Stationary tube cultures of L cells were incubated for 22 hr with interferon, 5 units/ml at 37°C . Cultures along with appropriate control cultures were then washed three times and refed with CS-free medium to stop cell division [17]. Groups of four tubes were selected and incubated for 0, 14, 24, 48 or 72 hr. At the indicated intervals, one group was inoculated with 10^3 p.f.u. VSV in 1 ml medium. Simultaneously, another group was treated with $50\text{ }\mu\text{g/ml}$ poly I-poly C in the presence of $100\text{ }\mu\text{g/ml}$ DEAE-dextran. After 1 hr incubation at 37°C the cultures treated with poly I-poly C were thoroughly washed and refed with medium. The cultures infected with VSV were examined microscopically every day to follow the development of cytopathic (CP) effect. The corresponding yield of virus was determined at the same intervals. The supernatants of the poly I-poly C treated cultures were assayed for interferon content at 12 and 24 hr postinduction.

Results

Effect of cycloheximide on the antiviral and priming activity of interferon. Cycloheximide inhibited the multiplication of L cells and at the end of the investigation period the number of cells in the drug-treated cultures was two to three times less than in the controls. As expected [18] this cytotoxic effect was followed by inhibition of virus replication. However, virus yield in the treated cultures was not usually more than 1 log_{10} below the yield in the controls. The reduction of yield could have been due to the impaired cell metabolism and the presence of less cells. Cycloheximide markedly inhibited the antiviral effect of interferon. The titre of virus in cultures treated with inter-

Table I
Effect of cycloheximide on the development of antiviral effect of interferon

Pretreatment		Virus yields* (p.f.u./ml)
2 hr	22 hr	
—	—	1.1×10^7
—	Interferon	9.7×10^1
Cycloheximide	Interferon + Cycloheximide	1.0×10^5
Cycloheximide	Cycloheximide	1.2×10^6

* assayed at 48 hr postinfection

feron in the presence of cycloheximide was on the average, $4 \log_{10}$ higher than in those treated with interferon alone (Table I).

Cycloheximide pretreatment completely abolished the interferon production of L cells but did not inhibit a reduced rate of interferon production when the cells were primed simultaneous interferon pretreatment (Table II).

Table II

Effect of cycloheximide on the development of priming effect of interferon

Pretreatment		Interferon yield (unit/ml)	
2 hr	22 hr	12 hr	24 hr
—	—	16	128
—	Interferon	128	64
Cycloheximide	Cycloheximide + Interferon	8	8
Cycloheximide	Cycloheximide	<2	<2

Duration of antiviral resistance and primed state. The cytopathic effect could be observed only on the fourth day of infection if cultures were challenged with VSV immediately after interferon pretreatment. Antiviral resistance markedly decreased during the first 14 hr but then persisted almost at the same level throughout a subsequent 58 hr period. Only a slight stepwise fall in the degree of antiviral state could be observed between 14 and 72 hr, as indicated by the gradually decreasing differences in virus titre between control and interferon treated cultures (Table III).

Table III

Duration of antiviral resistance in interferon pretreated L cells

Time of infection*	Pretreatment	CP effect** and corresponding yield of virus (p.f.u./ml) at intervals after infection			
		24 hr	48 hr	72 hr	96 hr
0	Interferon	0 (1.0×10^1)	0 (6.2×10^1)	0 (1.6×10^3)	1 (2.1×10^5)
	—	2 (7.8×10^6)	4 (1.3×10^7)	NT	NT
14	Interferon	0 (2.0×10^3)	2 (2.9×10^6)	4 (1.4×10^7)	NT
	—	2 (7.5×10^6)	4 (3.7×10^7)	NT	NT
24	Interferon	0 (2.4×10^3)	2 (4.1×10^6)	4 (3.1×10^7)	NT
	—	2 (8.1×10^6)	4 (4.0×10^7)	NT	NT
48	Interferon	0 (7.2×10^3)	2 (7.1×10^6)	4 (2.9×10^7)	NT
	—	2 (8.0×10^6)	4 (1.3×10^7)	NT	NT
72	Interferon	0 (2.1×10^4)	3 (9.5×10^6)	4 (1.0×10^7)	NT
	—	2 (7.5×10^6)	4 (2.1×10^7)	NT	NT

* hours relative to the end of interferon pretreatment

** 0: No CP effect; 1: +; 2: ++; 3: +++; 4: ++++

NT = not tested

The titre of interferon in the fluids of cultures treated with the interferon inducer immediately after interferon pretreatment, at 12 hr was five to ten times more than that in the controls (priming effect). The primed state of cells disappeared or declined to a low level after 14 hr incubation in interferon-free medium, and it was abolished on further incubation (Table IV).

Table IV

Duration of primed state in interferon pretreated L cells

Time of induction*	Pretreatment	Yield of interferon (unit/ml) at intervals after induction	
		12 hr	24 hr
0	Interferon	128	64
	—	16	128
14	Interferon	16	128
	—	8	128
24	Interferon	16	128
	—	16	128
48	Interferon	16	128
	—	16	256
72	Interferon	16	256
	—	32	256

* hours relative to the end of interferon pretreatment

Despite of the lack of CS in the culture medium, a slight increase in cell number could be observed in cultures incubated for 48 or 72 hr after interferon pretreatment. This might explain the high interferon titres obtained in the 24 hr samples of these cultures.

Discussion

STEWART *et al.* [14] have shown that L cells exposed to interferon in the presence of inhibitors of protein synthesis become primed yet develop no virus resistance. In contrast, a previous study [9] carried out in a chick embryo fibroblast cell – Chikungunya virus system suggested that the integrity of protein synthesis was necessary for the development of the priming effect of interferon. Our results obtained in a system similar to that of STEWART *et al.* but using polyI–polyC as interferon inducer instead of MM virus, seem to confirm that protein synthesis was not needed for the development of priming. The fact that in the present work cycloheximide was administered at a concentration toxic for L cells and yet the priming effect of interferon was not abolished, favours the said conclusions. The dose of cycloheximide explains

why we obtained lower interferon yields in the cultures pretreated with interferon plus cycloheximide.

It has been demonstrated by several authors [17, 19, 20, 21] that cells exposed to interferon remain protected for a considerable period of time. However, we know of no report describing the different duration of antiviral resistance and primed state of cells in response to interferon. In the present study we found that, following an initial decline, antiviral resistance persisted at an almost constant level throughout a further 58 hr testing period. In contrast, the primed state of cells rapidly waned with time and was completely abolished after 24 hr incubation.

The present findings did not allow any conclusion regarding the biochemical background of the different effects of interferon. Nevertheless, the observed difference in the duration of antiviral resistance and of the primed state seems to offer further indirect evidence suggesting that these activities are based on different changes in cell metabolism.

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STUDY OF COLICINOGENIC FACTORS E1 AND V IN RECOMBINATION SYSTEM

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Summary. Colicinogenic factors *colE1-ML* and *colV2-K94* were examined in $Hfr \times F^-$ recombination system. *colV* appeared in 100% of *thr⁺leu⁺* and of *arg⁺* recombinants. The transfer of *colV* was not influenced by *colE1*, but with *colV* factor-containing recipient the transfer of *colE1* resulted in the appearance of *colE1⁺* characteristic in the cells of the two selected markers in about the same proportion. In reverse crosses $HfrC \times F^-PA309(\lambda)E1 + V +$ the *arg⁺* recombinants frequently contained *E1-V⁺* cells.

Colicinogenic (*col*) factors of certain colicinogenic strains can be transferred to non-colicinogenic cultures [1]. In *Escherichia coli*, two types of *col* factors can be distinguished: *colV* and *colII* (self-transmitting with sex factor activity) and *colE1* and *colE2* (uncapable of self-transmission) [2]. The latter are transferred from the donor to the recipient by the sex factor.

Recently, FODOR *et al.* [3] have reported that in $Hfrcol^- \times F^-col^+$ crosses *colE1-ML* exhibited an anomaly, as the selected chromosomal recombinants had lost their *colE1-ML* determinants in a considerable percentage. As different plasmids may show incompatibility [4], the aim of the present work was to examine the effect of a sex factor active colicinogenic determinant V-K94, on the behaviour of determinant *colE1-ML*.

Materials and methods

Bacterial strains are listed in Table I. Most of them originated from the collection of Professor L. ALFÖLDI, Szeged; strains 84Nx and CA-7 were supplied by Professor P. FRÉDERICQ, Liège. All *colE1⁺* strains were derived from strains *HfrE1⁺* and *F-PA309E1⁺*.

Culture media. Complete medium: NaH_2PO_4 , 3 g; K_2HPO_4 , 7 g; NH_4Cl , 1 g; NaCl, 8 g; Bacto-Tryptone (Difco), 10 g; glucose, 1 g; Bacto-Yeast Extract (Difco), 1 g; distilled water, 1000 ml; after adjusting the pH with 10% NaOH to 7.2, the medium was autoclaved at 121°C for 30 min. The solid variety of the medium was prepared by adding 1.5% Bacto-Agar (Difco).

Minimal medium M9: NH_2PO_4 , 6 g; K_2HPO_4 , 14 g; NH_4Cl , 2 g; NaCl, 1 g; distilled water, 1000 ml; pH 7.2, 121°C for 30 min. The medium was solidified with 1.5% Bacto-Agar. The following ingredients were added after separate sterilization: 40% (w/v) glucose, 5 ml; 1 M $MgSO_4$, 1 ml; 1% vitamin B₁, 4 ml. The minimal medium was supplemented with 25 µg of the amino acids required per ml; streptomycin was added at 1000 µg/ml.

Overlaying of the agar plates was made with 0.6% aqueous Bacto-Agar.

Recombination. The donor and recipient strains were precultured overnight, transferred to complete medium and shaken gently until the beginning of the exponential phase (10^8 /ml). The cultures were centrifuged and the deposit was resuspended in fresh liquid complete medium so as to give identical optical densities. To 0.5 ml of the donor culture 9.5 ml recipient culture were added and the mixture was shaken gently at 37°C for 90 min. Cell counts for

Table I
Bacterial strains

Strains	Relevant characteristics
<i>E. coli</i> K-12 derivates	
Hfr Cavalli	<i>met</i> ⁻ <i>str</i> -s; polarity of transfer: <i>leu thr arg his trp</i>
Hfr Cavalli/ <i>E1</i>	<i>col E1-r str-s</i>
Hfr Cavalli/ <i>V</i>	<i>col V-r str-s</i>
Hfr Cavalli/ <i>E1V</i>	<i>col E1V-r str-s</i>
Hfr <i>E1</i> ⁺	<i>col E1-ML met</i> ⁻ <i>ile</i> ⁻
Hfr Cavalli <i>V</i> ⁺	<i>col V-K94</i>
Hfr <i>E1</i> ⁺ <i>V</i> ⁺	<i>col E1-ML col V-K94</i>
Indicator	
Hfr Cavalli	<i>met</i> ⁻ <i>str-r</i>
Hfr Cavalli/ <i>E1</i>	<i>col E1-r str-r</i>
Hfr Cavalli/ <i>V</i>	<i>col V-r str-r</i>
F-PA 309 (λ)	<i>thr</i> ⁻ <i>leu</i> ⁻ <i>arg</i> ⁻ <i>his</i> ⁻ <i>trp</i> ⁻ <i>str-r</i>
F-PA 309 (λ)/ <i>E1</i>	<i>col E1-r</i>
F-PA 309 (λ)/ <i>V</i>	<i>col V-r</i>
F-PA 309 (λ)/ <i>E1V</i>	<i>col E1V-r</i>
F-PA 309 (λ) <i>E1</i> ⁺	<i>col E1-ML</i>
F-PA 309 (λ) <i>V</i> ⁺	<i>col V-K94</i>
F-PA 309 (λ) <i>E</i> ⁺ <i>V</i> ⁺	<i>col E1-ML col V-K94</i>
Other strains	
<i>E. coli</i> 84 Nx(VI-94)	(<i>col V</i>) derivate of K-94 <i>cys</i> ⁻ <i>trp</i> ⁻ <i>met</i> ⁻
<i>E. coli</i> CA-7	<i>col V1</i>

Symbols: *met* = methionine; *thr* = threonine; *leu* = leucine; *arg* = arginine; *his* = histidine; *trp* = tryptophan; *ile* = isoleucine; *str* = streptomycin; Nx = nalidixic acid; *col* = colicinogenic; *r* = resistance; *s* = sensitivity

both parent strains were determined before and after incubation on M9 minimal medium devoid of threonine and leucine and of arginine. Counterselection was performed with streptomycin (1000 μ g/ml). The proportion of recombinants was expressed in per cents of the donor cells.

Colicin production. The number of colicinogenic cells in a population was determined by the double-layer technique of FREDERICQ [5]. If it was necessary to differentiate *E1* and *V* colicins, the double indicator strain method [6] was applied using strain *HfrC str-r* (sensitive to both colicins) and strain *HfrC/V* (sensitive to *E1*, resistant to *V*) at a proportion of 10 : 1. After incubation at 37°C colonies producing only *colV* formed large turbid zones 20–24 mm in diameter, while around colonies producing *colE1*, zones with sharp edges about 8 mm in diameter appeared. Colonies producing both types of colicin were characterized by a small clear zone surrounded by a large turbid zone. In certain experiments *E1 col-r* and *V col-r* indicators were employed separately. If a given dilution contained more than 20 c.f.u., in order to avoid confluent zones, the colonies were stabbed on complete medium plates.

Colicin resistant mutants were isolated by subculturing colonies grown within the zone of inhibition. The derivatives were checked for stability by repeated testing.

Results

Colicin resistance did not influence the result of recombination. As the *colV1* factor could not be transferred from strain CA-7 to strain Hfr Cavalli, the *colV2* factor present in a derivative of strain K-94 was used.

From the mixture of young HfrC/V and 84Nx(VI-94) cultures, a HfrCV⁺ strain was isolated as soon as after 1 hr. This strain retained its *colV* factor in serial subcultures (Fig. 1).

In HfrCV⁺ × F⁻PA309/V crosses, recombinants *thr*⁺ *leu*⁺ and *arg*⁺ gained *colV*⁺ characteristic in 100%. After a 90 min mating the reisolated

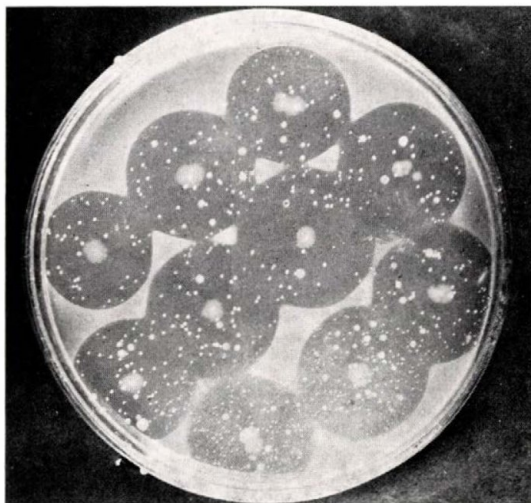


Fig. 1. Inhibition zones around HfrCV⁺ colonies on complete medium. Indicator: HfrC *str-r*

recipient cells contained colicinogenic cells in 20–30%. The recombinant colonies varied considerably in the size of inhibition zones (2–24 mm). Strain F-PA309V⁺ used for reversed conjugation, was isolated from the colicinogenic recipient cultures.

In HfrC/V × F⁻PA309V⁺ crosses both *thr*⁺*leu*⁺ and *arg*⁺ recombinants acquired *colV*⁺ characteristic in 100%. Donor cells reisolated at the end of the experiment had acquired *colV* factor from the recipient cells in 60–80%. The *colV* factor present in the donor or recipient cells did not influence the normal course of conjugation: the recombination proportions were 10–20% for *thr*⁺ *leu*⁺, and 1–2 for *arg*⁺.

In HfrCV⁺ × F⁻PA309EI⁺ crosses recombinants *thr*⁺*leu*⁺ and *arg*⁺ always contained the EI factor of the recipient; the *colV* factor of the donor also showed a 100% transfer. In this conjugation, the number of *arg*⁺ recombinants decreased considerably (Table II). The proportion of *arg*⁺ recombinants was less than 1% in all experiments, while the incidence of *thr*⁺*leu*⁺ recombinants showed a usual value. Reisolated recipient strains were found to have become EI⁺V⁺ (doubly colicinogenic) in 20–30%. The doubly colicinogenic cultures were isolated from these crosses. Double colicinogenicity allowed a re-

transfer of the *colE1* factor from F^- cells to Hfr Cavalli by the aid of *colV* factor.

In $HfrE1^+ \times F^-PA309V^+$ crosses recombinants with both *thr*⁺*leu*⁺ and *arg*⁺ markers acquired the *colV* factor in 100%. The transfer of *colE1* was approximately of the same frequency for *thr*⁺*leu*⁺ and *arg*⁺ recombinants (Table III). The recombination proportion between the two markers was as usual, but the values for the *thr*⁺*leu*⁺ marker showed a fairly high deviation in different experiments. Reisolated donor and recipient cells were shown to have acquired *col* factor from the counterpart parent in 11–28 and 3–12%.

In $HfrE1^+V^+ \times PA309/E1V$ crosses, at a usual recombination proportion the transfer of *E1* factor to *thr*⁺*leu*⁺ markers was lower (9.9%) and to *arg*⁺ marker higher (64.1%) as compared to a single transfer of *E1* (Table IV). Transfer of *colV* to the recombinants was 100%. At contransfer of the two *col* factors, the diameter of *colV* inhibition zones for the recombinant colonies

Table II

Hfr Cavalli V⁺ × *F*[−]*PA309E1*⁺

Experiment	Cells/ml		<i>thr</i> ⁺ <i>leu</i> ⁺ recombinants		<i>arg</i> ⁺ recombinants	
	Donor	Recipient	× 10 ³ /ml	Frequency, per cent	× 10 ³ /ml	Frequency, per cent
46	8 × 10 ⁸	4.5 × 10 ⁸	8100	20.2	35	0.08
51	5 × 10 ⁸	2 × 10 ⁸	8100	20.2	35	0.08
54	8 × 10 ⁸	4 × 10 ⁸	10085	27.1	264	0.66
113	2.9 × 10 ⁸	3.1 × 10 ⁸	3333	22.2	90	0.6
126	3.72 × 10 ⁸	1.95 × 10 ⁸	4540	24.4	18.3	0.09

Table III

HfrE1⁺ × *F*[−]*PA309V*⁺

Ex- peri- ment	Cells/ml		<i>thr</i> ⁺ <i>leu</i> ⁺ recombinants			<i>arg</i> ⁺ recombinants		
	Donor	Recipient	× 10 ³ /ml	per cent	<i>colE1</i> transfer*, per cent	× 10 ³ /ml	per cent	<i>colE1</i> transfer* per cent
50	5.6 × 10 ⁸	2.2 × 10 ⁸	1680	6.2	174/257=68.1	986	3.4	148/251=59.3
70	4.0 × 10 ⁸	1.2 × 10 ⁸	4593	22.7	23/ 45=51.1	933	4.6	19/ 36=52.7
92	2.5 × 10 ⁸	1.2 × 10 ⁸	210	1.6	23/ 63=36.5	73	0.58	45/135=33.3
94	2.3 × 10 ⁸	1.4 × 10 ⁸	114	0.9	117/340=34.4	53	0.46	51/143=35.6
115	2.2 × 10 ⁸	3.8 × 10 ⁷	1246	11.1	87/230=37.8	1848	1.6	32/109=29.3
119	2.8 × 10 ⁸	1.6 × 10 ⁸	6340	45.2	126/220=57.5	1490	10.6	114/210=52.1
			Total		550/1155=47.5			409/884=46.3

* No. of *colE1*⁺ colonies
No. of colonies examined = per cent

was definitely smaller than usually (6–8 mm *versus* 20–24 mm). As inhibition zones for *colV* frequently interfered with those for *colE1*, the two kinds of colicin could be distinguished safely only by parallel inoculation and use of the *col-r* counterpart indicator.

In *HfrC/EIV* $\times$ *PA309E1* $+V^+$ reverse crosses the *colV* factor appeared in the recombinants in 100%, while the *colE1* factor had been "lost" in part of the recombinants (Fig. 2). In the latter recombinants (*colE1* $-V^+$) *colE1*

Table IV

Hfr Cavalli E1+V+ $\times$ F-PA309EIV

Ex- peri- ment	Cells/ml		<i>thr⁺ leu⁺</i> recombinants			<i>arg⁺</i> recombinants		
	Donor	Recipient	$\times 10^3$ / ml	per cent	<i>E1+V+</i> transfer*, per cent	$\times 10^3$ / ml	per cent	<i>E1+V+</i> transfer*, per cent
68	1.54×10^8	2.19×10^8	3086	40.0	6/222=2.7	273	3.5	120/198=62.1
74	4.13×10^8	2.41×10^8	5247	25.4	16/100=16	1760	8.5	121/200=60.5
76	2.88×10^8	2.34×10^8	650	4.5	18/100=18	244	1.7	77/108=71.3
77	2.88×10^8	4.8×10^8	890	6.1	9/100=9	317	2.2	101/138=73.1
107	1.6×10^8	1.32×10^8	2400	30.0	23/200=11.5	430	5.3	122/200=61
Total					72/722=9.9			541/844=64.1

* No. of *E1+V+* colonies
No. of colonies examined = per cent

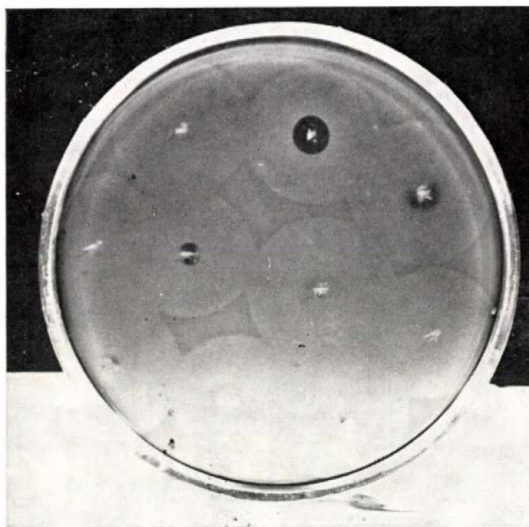


Fig. 2. *HfrC/EIV* $\times$ *PA309E1* $+V^+$. Inoculated from *arg⁺* recombinant colonies onto complete medium and overlayers with mixed indicator cultures after 24 hr incubation at 37°C:
colE1+V+ and *colE1-V+* colonies

Table V
Behaviour of colEI and colV in recombination systems

Crosses	Markers	col ⁺ per cent		
		V ⁺	EI ⁺	V ⁺ EI ⁺
Hfr Cavalli V ⁺ × F-PA 309/V	<i>thr leu</i>	100	—	—
	<i>arg</i>	100	—	—
Hfr Cavalli /V × F-PA 309 V ⁺	<i>thr leu</i>	100	—	—
	<i>arg</i>	100	—	—
Hfr EI ⁺ × F-PA 309/EI	<i>thr leu</i>	—	28.6	—
	<i>arg</i>	—	52.4	—
Hfr Cavalli /EI × F-PA 309 EI ⁺	<i>thr leu</i>	—	94	—
	<i>arg</i>	—	78	—
Hfr EI × F-PA 309 V ⁺	<i>thr leu</i>	100	—	47.5
	<i>arg</i>	100	—	46.3
Hfr Cavalli V ⁺ × F-PA 309 EI ⁺	<i>thr leu</i>	100	—	100.0
	<i>arg</i>	100	—	100.0
Hfr EI ⁺ V ⁺ × F-PA 309/EIV	<i>thr leu</i>	100	—	10.0
	<i>arg</i>	100	—	64.1
Hfr Cavalli/EIV × F-PA 309 EI ⁺ V ⁺	<i>thr leu</i>	100	—	75.5
	<i>arg</i>	100	—	8.0

production could not be induced either with UV irradiation or with mitomycin C. "Loss" of *colEI* factor seldom occurred in *thr*⁺*leu*⁺ recombinants but was very frequent in *arg*⁺ recombinants. The exact proportion of EI⁺V⁺ and EI⁻V⁺ colonies was not estimated, since *colEI* inhibitions frequently showed a transient form. Sometimes they appeared as cap-like semilunar areas beside the edge of the colonies. Subsequent testing of isolates from such colonies contained both *colEI*⁻V⁺ and *colEI*⁺V⁺ segregants. After further subculturing, the derivatives of the former were always EI⁻. Reisolated recipient colonies remained EI⁺V⁺ (double colicinogenic). The behaviour of *colV* and *colEI* factors in the recombination system is summarized in Table V.

Discussion

The *colV2* factor in itself has a sex factor character, being responsible for the transfer of the chromosomal segment. If strain V2-K94 was employed as donor, 95% of the recombinants acquired colicinogenicity [7]. KAHN and HELINSKI [8] in transferring *colV2* to F⁺ and F⁻ bacteria, found that F⁺ cells had frequently lost their *colV*⁺ characteristic, while F⁻ cells stably retained colicinogenicity. No similar incompatibility was observed for Hfr strains. Hfr strains acquired the *colV2* factor and the HfrV⁺ derivatives retained all their donor properties [7-9].

The HfrV⁺ strain used in the present experiments behaved similarly. V colicinogenicity appeared in 100% of the recombinants of any *colV* parent strain. The presence of *colEI* factor in the same culture or in the counterpart parent strain did not influence the behaviour of *colV*.

The *colE1* factor exerts no transfer capacity but if associated with sex factor-carrying cells it becomes transferable. The *colE1* factor in *E. coli* K-30 is mobilized by the aid of the F-like *colV3* [6].

The *col* factor of *E. coli* MLE1⁺ could be rendered suitable for genetic experiments if the *colE1* determinant had previously been transferred to the Hfr strain. In HfrE1⁺ × F⁻PA309/E1 crosses the transfer of *colE1* was more frequent to the proximal than to the distal marker. The presence of *colV* in the recipient influenced the transfer of *colE1* as the cells for the two markers acquired *colE1*⁺ characteristic in about the same proportion (Table III).

In HfrC × F⁻E1⁺ and in HfrCV⁺ × F⁻E1⁺ crosses there was a definite decrease of *arg*⁺ recombinants as compared to HfrC × F⁻E1⁺V⁺ conjugations. The reason for this finding is not known; it might be in connection with lethal zygotis [10]. FREDERICQ [11] failed to show a transfer of *colE1*⁻ characteristic of F⁺*colE1*⁻ × F⁻E1⁺ crosses. In the present experiments, similar reverse conjugations, especially HfrC × F⁻E1⁺V⁺ crosses, yielded *colE1*⁻ *arg*⁺ recombinants.

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BACTERIAL CONTAMINATION AND SPARING HEAT TREATMENT OF MOTHER'S MILK

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Summary. Heat inactivation of mother's milk was performed with the purpose to establish the optimum heat exposure which would kill all contaminating microorganisms without reducing the antibody content. After treatment at 65°C for 30 min, non-sporogenic live bacteria could no longer be demonstrated in the milk even with enrichment methods, but the titre of antibodies to a serogroup O 55 : K59(B5) *Escherichia coli* strain used as model did not change.

It was shown earlier that the immunoglobulins present in mother's milk are not absorbed from the infant's intestine [1]. In view of this the importance of breast feeding has been questioned. Recently, however, an increasing incidence of bacterial and fungal infections has been observed in the obstetric wards [2–5] and interest has again been centred on feeding with mother's milk. Analyses with modern immunological methods [6–8] showed that the main immunoglobulin component of the colostrum is IgA, occurring at a concentration of 20–40 mg/ml which, however, falls to about 1 mg/ml within a few days after delivery [9, 10]. The so-called secretory IgA present in mother's milk is more resistant to pH changes and proteolytic enzymes than serum IgA and it is also found in the stools of babies fed with mother's milk [11, 12]. The protective effect of mother's milk against enteric infections has widely been studied [13–18].

Untreated mother's milk becomes contaminated by bacteria or fungi depending on the mode of collection. KLINGE [19] reported that 84% of 51 milk samples originating from 38 women were contaminated by faecal bacteria. Similar observations made in Hungary [20] led to the recommendation that mother's milk collected for distribution should be heat-treated at 65°C for 30 min if its germ count reaches a certain level.

The present study has been carried out to assess the efficiency of treatment at various temperatures against the bacteria most frequently occurring in mother's milk and to determine the impact of heat inactivation on milk antibody content. An attempt was therefore made at the elaboration of a procedure suitable to kill non-sporogenic bacteria without affecting the antibodies in milk.

Materials and methods

Media. The following media were used in addition to those common in food bacteriology [21]: Kessler-Swenaton's medium in Durham tubes, Litsky-Mallman medium [22], Hugh and Leifson's O/F medium [23, 24], Polonyi's milk agar [25] for study of total germ count and proteolytic bacteria. The milk agar was prepared as follows: standard meat agar was liquefied in a water bath, cooled to 45°C and skim milk of 7.0 SH acid grade was added to it at a ratio of 5:1. The ready medium is wax-coloured, allowing an easy inspection of colonies; thus proteolytic bacteria are readily differentiated from the rest by the transparent halo which surrounds them.

Medium for gel adsorption antibody assay. Common broth (20 ml in Erlenmeyer flask) to which 0.25 mCi $\text{H}_2\text{H}^{32}\text{PO}_4$ was added before use.

Diluting fluid. A 1:10 mixture of Tris buffer (pH 7.5, 0.05 M) and saline.

Absorbent. Aluminium hydroxide, $\text{Al}(\text{OH})_3$ C_γ, Behring was suspended in distilled water so as to be equivalent to 1 g Al_2O_3 /100 ml. The suspension was diluted 1:4 with distilled water for use in the experiments.

Processing of milk samples for demonstration and quantitative determination of bacteria. Milk samples: (a) pooled mother's milk collected over 24 hr and stored at 4°C at the Mother's Milk Collection Station of Szeged from individual contributions withdrawn by donors at home; (b) pooled mother's milk freshly withdrawn in the obstetric ward of a hospital. The samples were processed according to the standard prescriptions of the National Institute of Food and Nutrition [25] and certain special media were additionally employed to provide optimum conditions of isolation and identification of all bacteria present. The samples were diluted in saline and inoculated into various media. The methods employed for determination of the live germ count allowed the demonstration of 1, 10 or 20 germs/ml. Enterococci were differentiated on the basis of SHERMAN's criteria [26] and *Pseudomonas aeruginosa* was identified as proposed by LÁNYI [27] and PINTÉR and BENDE [24].

The antigen for gel adsorption antibody assay was prepared by inoculating one loopful of an *Escherichia coli* O 55 : K59(B5) culture into the appropriate medium which was then incubated overnight at 37°C. Next morning the cultures were centrifuged and the sediment was washed with the diluting solution several times until the radioactivity of the supernatant was found to be negligible in comparison with that calculated for a similar dilution of the sediment (below 5%). The sediment was reconstituted so as to give a scintillation count of approx. 10,000/ml/min, and this bacterium suspension was used for antibody assay. The suspension can be stored at -4°C for several days, but the cells should be washed again before each use until the activity of the supernatant has decreased to the value given above.

Phage typing. *Staphylococcus aureus* and *P. aeruginosa* isolates were phage typed according to standard methods [28, 29].

Heat inactivation. For this purpose, two experimental schemes were employed. In the first, 10 ml of the test material was pipetted into each tube and the inactivation temperature was increased at 5°C steps (range: 65–80°C). In the second instance, about 5 ml of the test material was used per tube and the temperature increased at 2°C steps (range: 60–70°C). Fluctuation of the water bath temperature was $\pm 1^\circ\text{C}$.

Quantitative determination of antibodies to *E. coli* O55 in samples of mother's milk. The procedure employed was a modified version of a method described earlier [12, 30]. The working principle was that binding of antibody molecules to the surface of bacteria alters the latter's capacity for adsorption to aluminium hydroxide gel. The antibody-containing substance was diluted in serial steps to find the highest dilution still producing a measurable increase of bacterial adsorptivity as compared to controls. The bacteria used as antigen were labelled with ^{32}P , thus the proportion of binding to the adsorbent could be established by activity assay. The milk samples used for antibody assay were stored at -20°C; immediately before the experiment they were thawed and centrifuged at 5000 rpm for 25 min to remove fat. The de-fatted samples were diluted in twofold serial steps, in 0.5 ml volume; 0.5 ml of the *E. coli* O55 suspension was then added to each dilution and the mixture was incubated at 37°C for 3 hr. After incubation, the bacterium-whey mixture was centrifuged at 5000 rpm for 10 min, the sediment was washed in 5 ml diluting solution, centrifuged again at 5000 rpm for 15 min and the final sediment was reconstituted in 1 ml diluting solution. In the control series, diluting solution was used instead of milk. One ml $\text{Al}(\text{OH})_3$ gel was added to each tube, which was allowed to stand at room temperature for 20 min, then centrifuged at 1000 rpm for 2 min. The supernatants (bacteria unadsorbed) were assayed for radioactivity. The numerical value of the dilution step at which radioactivity was 50% of that measured in the control, was regarded as the antibody titre.

Results

Samples submitted over a period of two months were used for the detailed bacteriological analysis of mother's milk. First, the bacterial species most frequently occurring in the untreated supplies of the Mother's Milk Collection Station were identified.

Table I

Bacteria isolated from mother's milk

No. of mother's milk specimens tested, 59

Organisms	No. of specimens	Per cent
Proteolytic bacteria	20	33.9
Coliforms	32	54.2
<i>E. coli</i> I	5	8.5
<i>S. aureus</i>	22	37.3
Enterococci	6	10.2
<i>P. aeruginosa</i>	11	18.6
<i>Serratia marcescens</i>	2	3.4
<i>Alcaligenes faecalis</i>	1	1.7
<i>Achromobacter lwoffii</i>	3	5.1
<i>Achromobacter anitratus</i>	4	6.8
<i>Aeromonas</i>	3	5.1

As can be seen from Table I, the order of frequency of mixed infections was coliforms, *S. aureus*, proteolytic bacteria, *P. aeruginosa* and enterococci.

E. coli was found in 8.5% of the samples, indicating a faecal contamination of mother's milk. The frequency of Gram negative non-fermenting bacteria was also high and at such proportions they may constitute a source of infection [31].

The result of phage typing of *S. aureus* and *P. aeruginosa* strains isolated from pooled mother's milk supplied by private donors is shown in Table II.

Staphylococci were found in 45%, pseudomonads in 5.4% of the samples. The most frequent staphylococcal phage type, encountered in 82% of the cases, corresponded to the nosocomial strains. In a few cases a bovine phage type was demonstrated, whose presence may have been due to the addition of cow's milk to mother's milk. As to pseudomonas, all typable strains belonged to one and the same phage type.

In the second experiment, a milk sample highly contaminated with bacteria was treated at various temperatures to determine the heat exposure which would kill all live bacteria without notably reducing the titre of milk antibodies to *E. coli* O55, which served as the experimental model.

As can be seen from Table III, after heat-treatment at 65°C, no bacteria could be isolated from the milk.

The influence of heat treatment on milk antibody level was estimated from titre changes of antibodies to *E. coli* O55. This was an independent experiment and its results are summarized in Table IV.

Table II

Phage types of S. aureus and P. aeruginosa strains isolated from 184 mother's milk specimens over a period of one year

<i>S. aureus</i>			<i>P. aeruginosa</i>	
Group	No.	Phage type	No.	Phage type
III	52	53/77/83A/85	8	21/68/119x/
Bovine	7	heterologous		352/1 ₁₃ /1 ₂₁
Other	4	heterologous		
—	26	untypable	2	untypable
Total	89		10	

Table III

Changes in bacterial content of pooled native mother's milk after heat treatment in the range from 65 to 80°C

Temperature of inactivation (°C) for 30 min	Bacterial count/ml					
	Total germ count	Proteolytic	Coliforms	<i>E. coli</i> I	<i>S. aureus</i>	Enterococci
Untreated	20 000 000	1 000 000	11 100 000	900 000	1850	50 000
65	< 20	< 20	< 10	< 10	< 1	< 1
70	< 20	< 20	< 10	< 10	< 1	< 1
75	< 20	< 20	< 10	< 10	< 1	< 1
80	< 20	< 20	< 10	< 10	< 1	< 1

No notable changes in antibody level occurred in four milk samples after treatment at 65 and 70°C for 30 min. To obtain more information on the conditions of heat inactivation, the temperature was increased at 2°C steps and the amount of the sample was reduced to 5 ml. Under such conditions the source of error associated with the uneven warming up of the sample was negligible. Results are shown in Table V.

Both *S. aureus* and enterococci were demonstrable by enrichment methods after heating at 60°C for 30 min, but no live bacteria were found if the temperature was increased to 62°C. This was in fair agreement with our previous findings, but allowed a more precise estimate of the narrow critical temperature range (68–70°C) at which the antibodies become abruptly inactivated.

Table IV

Antibody levels to *E. coli* O55 : K59(B5) in mother's milk samples after heat inactivation

Temperature of inactivation (°C) for 30 min	Reciprocal values of antibody titres measured in various mother's milk samples			
	1	2	3	4
Room temperature (25°C)	>1024	256	32	70
60	>1024	NT	NT	NT
65	NT	180	14	75
70	800	NT	NT	45
75	NT	<2	<2	<2
80	<2	<2	<2	<2

NT = not tested

Table V

Live bacterial count and antibody level to *E. coli* O55 : K 59(B5) in samples of mother's milk after heat inactivation

Temperature (°C) of inactivation for 30 min	Bacterial count/ml							Reciprocal value of antibody titre
	Total germ count	Proteolytic	Coliforms	<i>S. aureus</i>	Enterococci	<i>P. aeruginosa</i>	<i>Achromobacter anitratus</i>	
Room temperature (25°C)	20 000 000	2 000 000	3 600 000	1 to 9	6 400	9 500	80 000	600
60	<20	<20	<10	1 to 9	1 to 9	<10	<10	580
62	<20	<20	<10	<1	<1	<10	<10	750
64	<20	<20	<10	<1	<1	<10	<10	512
66	<20	<20	<10	<1	<1	<10	<10	1024
68	<20	<20	<10	<1	<1	<10	<10	810
70	<20	<20	<10	<1	<1	<10	<10	<2

Table VI

Antibody level to *E. coli* O55 : K59(B5) in mother's milk with increased bacterial content

Sample of mother's milk	Bacterial count/ml						Reciprocal value of antibody titre
	Total germ count	Proteolytic	Coliforms	<i>S. aureus</i>	Enterococci	<i>P. aureginosa</i>	
Freshly taken	22 000	<20	<10	4 650	<1	<10	600
After infection and 48-hr incubation at 25°C	4 800 000	800 000	4 000 000	360 000	160 000	110 000	30

Next it was studied whether at common degrees of bacterial contamination the enzymic activity of bacteria would notably reduce the milk antibody levels. Samples freshly collected in an obstetric ward were pooled, part of the pool was stored in the refrigerator, and part was infected with a milk sample of high bacterial content and incubated at room temperature for 48 hr. Subsequently, both pools were examined for bacterial content and antibody levels. As seen from Table VI, the antibody content of the infected sample fell to 5% of that measured in the refrigerated non-infected sample, owing in all probability to bacterial enzymic activity.

Discussion

Mother's milk is an important source of antibodies to enteric pathogens and other infective microorganisms such as streptococci and pneumococci, and to various viruses. Its anti-microbial and antiviral activity is, however, due not only to humoral, but also to cellular [32] and enzymic factors [33]. BULLEN *et al.* [34-36] demonstrated that the iron-binding protein lactoferrin, occurring in mother's milk at high concentrations (2-6 mg/ml) exerts a distinctive bacteriostatic effect on *E. coli* O111, which is potentiated by the presence of specific antibodies, but terminated by saturation of the milk with iron. Heat treatment at 65°C affects neither transferrin, nor lactoferrin.

The circumstance that under the existing conditions of collection, the hygienic conditions at obtaining and transport of mother's milk by the individual donors cannot be controlled appropriately and the fact that owing to general shortage, mother's milk of high bacterial contents cannot be discarded, call for new methods of treatment and preservation. SURÁNYI *et al.* [37] recommended a freeze-drying of batches whose germ counts are below the tolerated limits. Comments on the hygienic aspects of this procedure are beyond the scope of the present paper, but certain technical details of heat treatment deserve special mention.

An even heating of the milk is imperative, especially when large quantities are inactivated. Consideration should also be given to the fact that although heat treatment inactivates the toxin-producing bacteria, it does not destroy their heat-stable toxic products (enterotoxin). Assessment of the bacteriological quality of the milk before heat treatment and an appropriate selection of the batches after heat treatment is therefore important.

In view of the present findings, heat inactivation studies should be extended to pathogenic agents other than bacteria (*e.g.* fungi, hepatitis B virus or antibody).

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MICRODIAGNOSTIC METHOD FOR THE RAPID IDENTIFICATION OF BACTERIA

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Summary A rapid, non-laborious microdiagnostic method has been elaborated for laboratories with limited facilities. By the use of 13 different biochemical reactions the time of examination decreased to one third to one tenth of that required by the classical methods. A total of 49,830 comparative examinations using 4670 bacterial strains showed a 1.8% difference from the results obtained with the standard methods. The H_2S indole, nitrite, urease, glucose, sucrose, maltose, oxidase and catalase reactions were more sensitive, the gelatin, lactose and mannitol reactions were less sensitive, than the corresponding classical tests.

In view of the increasing importance of reliable bacteriological diagnosis, it seemed desirable to elaborate a rapid and simple method allowing a proper differentiation of bacteria in peripheral field or mobile laboratories with minimum facilities.

As a first step of this study, the papers of BRONFANBRENNER and SCHLESINGER [1], KNOX [2], SZITA [3], SNYDER [4], SANDERS *et al.* [5] and of WEAVER's team [6–13] were used as a basis for the choice of the reagents. The method was later refined [14], the reagents were patented and are now manufactured by Reanal, Budapest.

Materials and methods

Media and reagents. *Urease production and carbohydrate fermentation.* Urea, glucose, lactose, sucrose, maltose or mannitol, 32 g; NaCl, 6 g; Na_2HPO_4 1.6 g; KH_2PO_4 , 0.4 g; 1% bromthymol blue solution, 4 ml; 2% acid fuchsin solution, 4 ml; nutrient broth to 200 ml; pH, for urease test 6.6, for carbohydrate fermentation test, 7.6; sterilized by filtration.

Oxidase and catalase. Peptone (Richter), 10 g; peptone (Witte), 5 g; beef extract, 7 g; brewer's yeast extract, 20 ml; NaCl, 10 g; Na_2HPO_4 , 7.5 g; KH_2PO_4 , 3 g; $MgSO_4$ 1.5 g; distilled water to 200 ml; pH 7.2; 110°C 20 min. Oxidase reagent: N-N-dimethyl-p-phenylenediamine dihydrochloride, powdered. Catalase reagent: 5% aqueous H_2O_2 .

Gelatin. Gelatin, bacteriological, 40 g; NaCl, 2.5 g; Na_2HPO_4 , 1.5 g; KH_2PO_4 , 1 g; distilled water to 200 ml; pH 7.2; 110° 20 min. Reagent: ninhydrin, 1 g; ethanol, 9 ml.

H_2S production. Peptone (Richter), 8 g; peptone (Witte), 2 g; dextrin, 4 g; beef extract, 10 g; brewer's yeast extract, 20 ml; 1% lead acetate solution, 2.4 ml; 20% Na_2SO_3 solution, 8 ml; 2% $FeSO_4$ solution, 20 ml; DL-cysteine, 0.4 g; distilled water to 200 ml; pH 7.6; sterilized by filtration.

Indole production. DL-tryptophan, 2 g; N NaOH 10 ml; nutrient broth to 250 ml; pH 7.6; 110° 20 min. Indole reagent: filter paper soaked in p-dimethylamidobenzaldehyde, 5 g; methanol, 150 ml; concentrated phosphoric acid, 100 ml.

Nitrite production. Glucose, 6 g; 10% $NaNO_3$ solution, 20 ml; 10% KNO_3 solution, 20 ml; nutrient broth to 200 ml; pH 7.4, sterilized by filtration. Nitrite reagent powder: alpha naphthylamine, 0.5 g; sulphanilic acid, 5 g; tartaric acid, 94.5 g.

Microtest set. The media and reagents are distributed into 2 ml plastic ampoules. The set contains disposable and non-disposable plastic plates, dilution blocks and a lancet for the distribution of powders (Fig. 1).

Technique of microtests. 1. The colony to be examined is precultured on agar slant for 4 hr (fastidious organisms on blood agar slant for 4–18 hr).

2. The agar slant culture is suspended in distilled water (usually in 2 ml), to give 10^7 – 10^8 cells/ml.

3. The suspension is pipetted into the wells of the plates at 0.1 ml portions, then 0.02 ml amounts of the concentrated media are added (2 drops from the plastic ampoules). The microculture is incubated at 37°C for 4 hr (with fastidious organisms for 4–10 hr). If a special environment is needed, disposable plates with the microculture are placed in air-tight Petri dishes; CO₂ atmosphere is produced using hydrochloric acid and sodium carbonate, anaerobic conditions are produced using pyrogalllic acid and sodium hydroxide (Fig. 2).

4. Before reading the oxidase, catalase, gelatin, indole and nitrite reactions, the corresponding reagents are added to the microcultures.

Bacterial strains. For comparing the time needed for the rapid tests and the standard methods (Table I), a total of 1257 strains was tested, viz., 8 *Alcaligenes faecalis*, 79 *Bacillus* sp.,

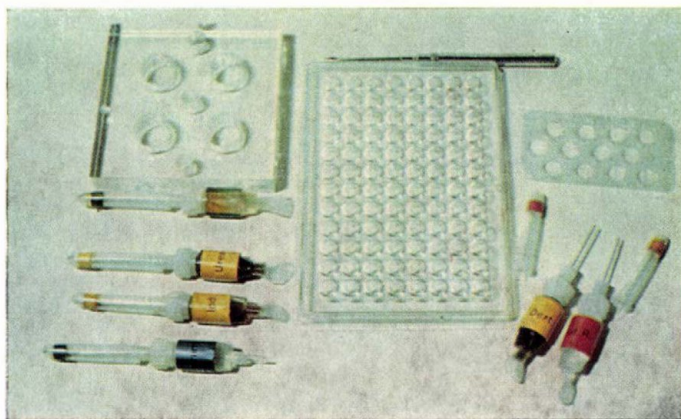


Fig. 1. Microdiagnostic set

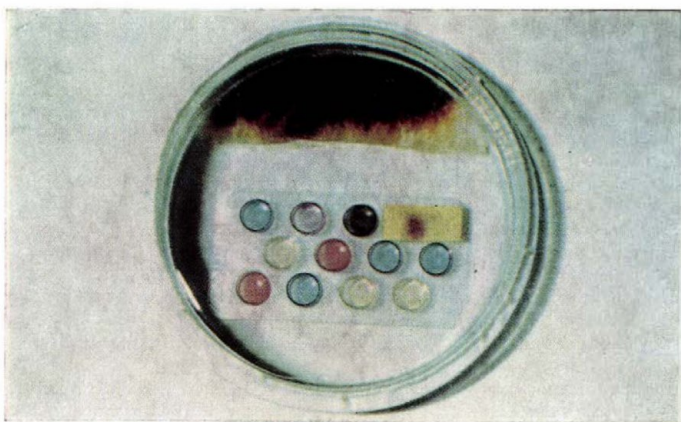


Fig. 2. Biochemical reactions of *Clostridium sordellii* in anaerobic microculture: urease +, gelatin +, H₂S +, indole +, nitrite —, glucose +, lactose —, sucrose —, maltose +, mannitol —, oxidase —, catalase —

6 *Brucella* sp., 67 *Citrobacter freundii*, 18 *Clostridium* sp., 33 *Corynebacterium* sp., 18 *Diplococcus pneumoniae*, 112 *Enterobacter* sp., 180 *Escherichia coli*, 31 *Neisseria* sp., 62 *Proteus mirabilis*, 18 *Proteus morganii*, 31 *Proteus rettgeri*, 49 *Proteus vulgaris*, 76 *Pseudomonas aeruginosa*, 29 *Pseudomonas fluorescens*, 82 *Salmonella* sp., 9 *Providencia* sp., 16 *Serratia marcescens*, 89 *Shigella* sp., 78 *Staphylococcus aureus*, 88 *Staphylococcus epidermidis*, 41 *Streptococcus faecium*, and 17 *Streptococcus liquefaciens* strains.

For comparing the sensitivity of the rapid tests and the standard methods, 3413 strains were tested; they are listed in Table II. Of the latter strains, 3360 had been isolated from routine material and 53 obtained from culture collections.

Results

Time needed for performing the microdiagnostic tests. For comparing the rapid tests with the standard method [15-19], 8879 parallel reactions were carried out with 1257 bacterial strains listed in "Materials and methods". The shortest time elapsing from the inoculation till the appearance of a well-defined reaction was regarded as the time needed for the test. The cultures were selected so as to represent not only the "prompt" but also the "delayed" reactions for all biochemical tests. Results are shown in Table I.

Table I

Time of positive reaction in standard tests and with microdiagnostic method

Reaction	No. of tests	Time of positive reaction, hr		
		Standard tests	Microdiagnostic method	
			without precultivation	with precultivation
Glucose	800	26	3	11
Lactose	800	36	4	16
Sucrose	800	36	4	14
Maltose	800	36	4	14
Mannitol	800	36	4	12
Urease	800	18	2	9
Gelatin	600	37	6	10
H ₂ S	800	40	4	8
Indole	800	50	2	12
Nitrite	800	26	3	8
Methyl red	800	30	4	10
Coagulase	137	28	3	10
Catalase	137	25	2	8
Total	8874	424	45	142
Average for one reaction		32.6	3.5	11.0

Table II
Difference in the results of

Organism	No. of strains	Microdiganostic test							
		Urease		Gelatin		H ₂ S		Indole	
		+	-	+	-	+	-	+	-
<i>Acinetobacter anitratus</i>	45	—	—	—	—	—	—	—	—
<i>Aeromonas liquefaciens</i>	20	—	—	—	—	1	—	1	—
<i>Alcaligenes faecalis</i>	20	—	—	—	—	1	—	—	—
<i>Bacillus anthracis</i>	9	—	—	—	1	—	—	—	—
<i>Bacillus cereus</i>	52	—	—	—	—	—	2	—	—
<i>Bacillus megaterium</i>	27	—	—	3	1	—	—	—	—
<i>Bacillus subtilis</i>	61	2	—	—	—	—	—	—	—
<i>Bacillus</i> other	18	3	2	4	3	—	—	—	—
<i>Brucella abortus</i>	6	—	—	—	—	2	—	—	—
<i>Brucella melitensis</i>	4	—	—	—	—	—	—	—	—
<i>Brucella ovis</i>	4	—	—	—	—	4	—	—	—
<i>Brucella suis</i>	8	—	—	—	—	2	—	—	—
<i>Citrobacter freundii</i>	173	2	—	—	—	—	—	—	—
<i>Clostridium botulinum</i>	2	—	—	—	—	—	—	—	—
<i>Clostridium histolyticum</i>	4	—	—	—	—	—	—	—	—
<i>Clostridium oedematiens</i> (I)	3	—	—	—	1	1	—	—	—
<i>Clostridium perfringens</i> (A)	5	—	—	—	1	—	—	1	—
<i>Clostridium sordellii</i>	12	1	—	—	—	—	—	1	—
<i>Clostridium sporogenes</i>	18	—	—	—	—	—	—	1	—
<i>Clostridium tetanomorphum</i>	19	—	—	—	1	—	—	1	—
<i>Clostridium</i> other	9	—	—	1	1	1	1	—	—
<i>Corynebacterium diptheriae</i>	2	—	—	—	—	—	—	—	—
<i>Corynebacterium hoffmannii</i>	16	—	—	—	—	—	—	—	—
<i>Corynebacterium liquefaciens</i>	5	—	—	—	1	1	—	—	—
<i>Corynebacterium poinsettiae</i>	3	—	—	—	—	—	—	—	—
<i>Corynebacterium renale</i>	8	—	—	—	—	—	—	—	—
<i>Corynebacterium ulcerans</i>	4	—	1	1	1	—	—	—	—
<i>Corynebacterium xerose</i>	12	—	—	—	—	—	—	—	—
<i>Diplococcus crassus</i>	26	—	—	—	—	—	—	—	—
<i>Diplococcus pneumoniae</i>	41	—	—	—	—	—	—	—	—
<i>Edwardsiella tarda</i>	15	—	—	—	—	—	—	—	—
<i>Enterobacter</i> sp.	227	15	8	7	14	2	1	—	—
<i>Escherichia coli</i> var. comm.	212	—	—	—	—	—	—	3	—
<i>Escherichia coli</i> var. intermed.	161	—	—	—	—	—	—	5	—
<i>Escherichia coli</i> var. alc. dispar	72	—	—	—	—	—	—	—	—

standard tests and microdiagnostic tests

not conforming to standard method, no. of strains

Nitrite + —	Glucose + —	Lactose + —	Sucrose + —	Maltose + —	Mannitol + —	Oxidase + —	Catalase + —
— —	— —	— —	— —	— —	— —	1 —	1 —
— —	— —	2 —	3 —	3 —	3 —	1 —	— —
1 —	— —	— —	— —	— —	— —	1 —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	3 —	2 —	— —	— —	2 —
2 —	— —	1 1	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	3 —	— —	— —	1 2	— —	4 3
— —	— —	— —	— —	— —	— —	— —	1 —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	4 5	3 1	— —	— —	— 2
— —	— —	— —	— —	1 —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	2 —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— 1	— —	— —	1 —	1 —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	2 —
1 —	— —	— —	— —	— —	— —	— —	— —
1 —	— —	1 —	1 —	— —	— —	— —	1 —
— —	— —	— —	— —	— —	— —	— —	— —
3 —	— —	— —	— —	— —	— —	— —	2 —
1 —	— —	— —	1 —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
1 —	— —	— —	— —	— —	— —	— —	1 1
— —	— —	— —	— —	— —	— —	— —	— 1
— —	— —	— —	2 —	1 —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
1 —	— —	— —	— —	— —	— —	— —	— —
— —	— —	5 9	4 11	2 1	1 1	— —	10 3
— —	— —	— —	— —	2 —	— —	— —	— —
— —	— —	— —	4 3	— —	— —	— —	2 1
— —	— —	1 —	3 —	3 —	— —	— —	4 —

(Continued)

Organism	No. of strains	Microdiagnostic test							
		Urease		Gelatin		H ₂ S		Indole	
		+	-	+	-	+	-	+	-
<i>Erwinia</i> sp.	103	2	1	8	12	—	—	6	1
<i>Erysipelothrix insidiosa</i>	7	—	—	—	—	2	—	—	—
<i>Gaffkya tetragena</i>	82	—	—	—	—	—	—	—	—
<i>Hafnia alvei</i>	10	—	—	—	—	—	—	—	—
<i>Haemophilus influenzae</i>	6	1	—	—	—	—	—	—	—
<i>Haemophilus paraptussis</i>	4	—	—	—	—	—	—	—	—
<i>Klebsiella pneumoniae</i>	48	—	—	—	—	—	—	—	—
<i>Lactobacillus</i> sp.	26	—	—	—	—	—	—	—	—
<i>Leuconostoc</i> sp.	7	—	—	—	—	—	—	—	—
<i>Listeria monocytogenes</i>	15	—	—	—	—	—	—	—	—
<i>Micrococcus ureae</i>	51	—	—	1	—	—	—	—	—
<i>Micrococcus epidermidis</i>	59	1	—	2	3	—	—	—	—
<i>Micrococcus</i> other	64	2	1	3	4	—	—	—	—
<i>Moraxella</i> sp.	11	—	—	—	—	—	—	—	—
<i>Neisseria catarrhalis</i>	26	—	—	—	—	—	—	—	—
<i>Neisseria flava</i>	16	—	—	—	—	—	—	—	—
<i>Neisseria gonorrhoeae</i>	2	—	—	—	—	—	—	—	—
<i>Neisseria meningitidis</i>	4	—	—	—	—	—	—	—	—
<i>Neisseria sicca</i>	15	—	—	—	—	—	—	—	—
<i>Neisseria</i> other	2	—	—	—	—	—	—	—	—
<i>Pasteurella pestis</i>	6	—	—	—	—	—	—	—	—
<i>Pasteurella pseudotuberculosis</i>	14	—	—	—	—	1	—	—	—
<i>Pasteurella tularensis</i>	3	—	—	—	—	—	—	—	—
<i>Pasteruella ureae</i>	2	—	—	—	—	—	—	—	—
<i>Peptococcus aerogenes</i>	4	—	—	—	—	1	—	1	—
<i>Peptostreptococcus intermedius</i>	6	—	—	—	—	—	—	—	—
<i>Proteus mirabilis</i>	67	—	—	—	2	—	—	—	—
<i>Proteus morganii</i>	39	—	—	—	—	7	1	2	—
<i>Proteus rettgeri</i>	48	—	—	—	—	—	—	—	—
<i>Proteus vulgaris</i>	71	—	—	—	3	—	—	4	—
<i>Providencia</i> sp.	17	—	—	—	—	—	—	—	—
<i>Pseudomonas aeruginosa</i>	103	17	—	13	4	—	—	—	—
<i>Pseudomonas fluorescens</i>	28	—	—	—	—	—	—	—	—
<i>Pseudomonas pseudomallei</i>	3	—	—	—	—	1	—	—	—
<i>Pseudomonas putida</i>	2	—	—	—	—	—	—	—	—

not conforming to standard method, no. of strains

Nitrite + —	Glucose + —	Lactose + —	Sucrose + —	Maltose + —	Mannitol + —	Oxidase + —	Catalase + —
— —	— —	2 1	4 5	— —	3 5	— —	— —
1 —	— —	— —	— —	— —	— —	— —	— —
— —	— —	3 —	— —	— —	— —	— —	6 —
— —	— —	— —	1 —	— —	— —	— —	— —
1 —	— —	— —	1 —	1 —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	1 —
— —	— —	— —	5 2	3 1	— —	— —	— —
— —	— —	1 —	— —	— —	— —	— —	— —
— —	— —	1 1	1 —	2 1	— —	— —	1 1
— —	1 —	— —	— 1	1 1	— —	— —	— —
— —	— —	2 3	— 1	1 —	— —	— —	1 —
3 —	1 —	2 3	2 1	2 4	1 6	1 —	6 2
— —	— —	— —	— —	— —	— —	2 1	3 2
2 —	— —	— —	— —	— —	— —	— 1	1 1
— —	— —	1 1	— —	— —	— —	— 1	1 —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— 1	— —	1 —	— —
2 —	— —	1 —	1 —	1 —	— 1	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
1 —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	1 —	— —
— —	— —	— —	— —	— —	— —	— —	— —
1 —	— —	1 —	1 —	— —	1 —	— —	— —
— —	— —	— —	— —	— —	1 —	— —	— —
— —	— —	— —	3 —	— —	1 —	— —	3 1
— —	— —	— —	— —	— —	— —	— —	2 —
— —	— —	— —	— —	— —	— —	— —	1 —
— —	— —	— —	4 —	1 —	1 —	— —	4 —
— —	— —	— —	3 —	— —	— —	— —	— —
12 —	5 —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	1 —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —

(Continued)

Organism	No. of strains	Microdiagnostic test			
		Urease + —	Gelatin + —	H ₂ S + —	Indole + —
<i>Salmonella typhi</i>	18	— —	— —	4 —	— —
<i>Salmonella paratyphi-A</i> and <i>B</i>	10	— —	— —	1 —	— —
<i>Salmonella arizonae</i>	11	— —	3 1	— —	— —
<i>Salmonella other</i>	127	— —	2 3	2 1	— —
<i>Sarcina flava</i>	35	1 —	— —	— —	— —
<i>Sarcina ureae</i>	19	— 1	— —	— —	— —
<i>Sarcina ventriculi</i>	7	— —	— —	— —	— —
<i>Serratia marcescens</i>	49	— —	— 3	— —	— —
<i>Serratia other</i>	18	— —	1 2	— —	— —
<i>Shigella boydii</i>	38	— —	— —	— —	1 1
<i>Shigella dysenteriae</i> (1—8)	14	— —	— —	— —	1 —
<i>Shigella flexneri</i> (1—6)	85	— —	— —	— —	— —
<i>Shigella sonnei</i>	81	— —	— —	— —	— —
<i>Staphylococcus aureus</i>	88	11 2	3 7	— —	— —
<i>Staphylococcus epidermidis</i>	101	8 3	4 11	— —	— —
<i>Staphylococcus other</i>	21	2 —	3 —	1 —	— —
<i>Streptococcus durans</i>	41	— —	— —	— —	— —
<i>Streptococcus faecium</i>	68	— —	— —	— —	— —
<i>Streptococcus liquefaciens</i>	27	— —	— 3	— —	— —
<i>Streptococcus zymogenes</i>	33	— —	1 5	— —	— —
<i>Streptococcus viridans</i>	70	— —	— —	— —	— —
<i>Streptococcus other</i>	113	— —	— —	1 —	— —
<i>Vibrio cholerae</i>	24	— —	1 2	— —	— —
<i>Vibrio cholerae</i> var. <i>el-tor</i>	7	— —	— —	— —	1 —
<i>Vibrio</i> sp.	14	— —	— —	— —	— —
Total	3413	68 19	61 90	36 6	29 2
Discrepant reactions, total		87	151	42	31
Discrepant reactions, per cent		2.5	4.4	1.2	0.9

The average time needed for one standard test was 32.6 hr as compared to 3.5 hr for one rapid test. If the time of precultivation is added, the average for one rapid test was 11.0 hr.

Reliability of the microdiagnostic tests. A total of 40 956 parallel test was performed with 3413 strains. The cultures represented a wide range of genera and species. Results are presented in Table II.

not conforming to standard method, no. of strains

Nitrite + —	Glucose + —	Lactose + —	Sucrose + —	Maltose + —	Mannitol + —	Oxidase + —	Catalase + —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— 3	3 —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	3 1
1 —	— —	— —	— —	— —	— —	— —	1 1
— —	— —	— —	1 —	— 1	— —	— —	— —
2 —	— —	4 6	— —	— —	— —	— —	— —
— —	— —	2 1	1 2	2 2	1 1	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	3 1	— —	— —	— —	— —	— —
2 —	— —	— 1	— —	— —	5 7	— —	1 —
7 —	— —	— 3	— —	— —	3 5	— —	3 —
1 —	— —	— —	1 —	— —	— 1	— —	2 —
— —	— —	— —	3 1	— —	3 1	— —	— —
— —	— —	— —	1 1	— —	— 2	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— 1	— 2	— —	— —	— —	— —
— —	— —	2 1	2 2	1 3	1 —	— —	— —
— —	— —	— —	— —	— —	— —	— —	— —
— —	— —	— —	— —	1 —	— —	— —	— —
2 —	— —	1 —	— —	2 —	— —	1 —	— —
49 1	7 —	36 39	55 49	28 22	23 34	8 5	71 21
50	7	75	104	50	57	13	92
1.4	0.2	2.2	3.0	1.4	1.7	0.4	2.7

The results obtained for each strain with either the standard or the rapid test, were reproducible within the limits of experimental error. Reactions of fastidious organisms could be accelerated by adding inactivated ox serum to the microcultures. The colour reactions of various bacteria are shown in Fig. 3.

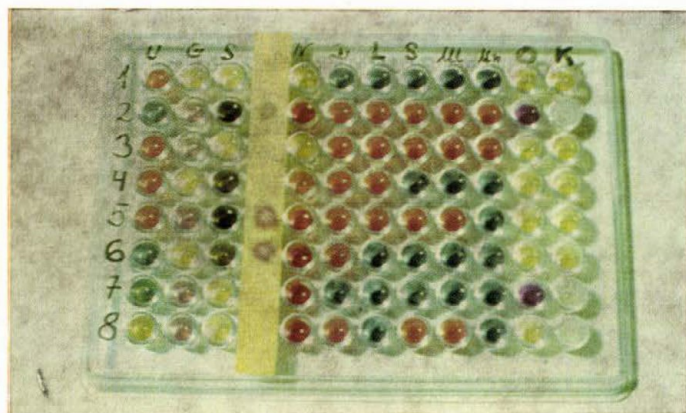


Fig. 3. Biochemical reactions in microcultures of various bacteria. Abbreviations: U = urease, G = gelatin, H_2S = sulphur hydrogen, I = indole, N = nitrite, D = glucose, L = lactose, S = sucrose, Ml = maltose, Mn = mannitol, O = oxidase, K = catalase. Line 1: all reactions negative; line 2: all reactions positive; line 3: *Streptococcus liquefaciens*; line 4: *Erysipelothrix insidiosa*; line 5: *Erwinia betivora*; line 6: *Morganella morganii*; line 7: *Pseudomonas aeruginosa*; line 8: *Bacillus cereus*

Discussion

In view of the low incidence (0.4–4.4%) of discrepancies between the microdiagnostic and standard tests, the former may be regarded as adequate for determining the biochemical reactions of important bacteria. The results indicated that the method was probably suitable for testing other reactions (fermentation of other sugars and alcohols, utilization of citrate, malonate and tartrate, decarboxylation of lysine and ornithine, deamination of phenylalanine, methyl red, ONPG, formazan, phosphatase, etc.). By the use of the rapid technique, the time of diagnosis is considerably shortened, to 1/3–1/10 of the time of the standard tests. On the average, the microdiagnostic method showed discrepancy of not more than 1.8% from the standard tests. The urease, H_2S , indole, nitrite and catalase reactions are detected especially rapidly, with the micromethod these reactions were positive in 3–4 hr even if with certain cultures the corresponding standard tests become positive not earlier than 5–6 days. The microtest for gelatin liquefaction proved less sensitive.

The microdiagnostic method, as compared to the standard tests, needs a small desk place (only $10 \times 130 \times 90$ mm plastic plates are used for 96 reactions), very small amounts of media (0.01 ml medium for one reaction) and is not laborious.

The microdiagnostic set is suitable for simple morphological and serological examinations to supplement the biochemical reactions.

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“PLEXIFORM VERMICELLAR ARRAYS” IN HUMAN CYTOMEGALOVIRUS-INFECTED WI-38 HUMAN EMBRYONIC FIBROBLAST CELLS

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Summary. Plexiform vermicellar arrays were seen in the nucleus of three out of four primary cytomegalovirus isolate-infected WI-38 cell cultures in a small percentage of infected cells (but never in uninfected cells). They do not appear to be related directly to CMV fabrication in these cells. The nature and origin of these structures remain to be elucidated.

Intranuclear plexiform vermicellar arrays (PVA's) were observed by SWANSON *et al.* [1] in a brain biopsy from a patient with acute herpes simplex encephalitis. The structures occurred in the nuclei of infected neurons, and were similar to the granular aggregates noted by MORGAN *et al.* [2] in HeLa cell nuclei infected with herpes virus hominis (HVH). Strikingly similar intranuclear PVA's were noted in WI-38 cells used for isolating human cytomegalovirus (CMV) from the urine (or organs) of patients who had received a renal allograft. Such intranuclear arrays have not been observed by previous investigators in cells infected by various strains of CMV in cell cultures [3–9], nor in cells infected by primary virus isolates [8, 10, 11]. To investigate the possibility of these being a CMV strain-related phenomenon, several WI-38 cell cultures, which had been used for primary isolation of CMV from patients with renal allograft, were examined to elucidate the relationship of these structures to virus multiplication.

Materials and methods

WI-38 cells obtained from Flow Laboratories, Bethesda, Maryland, were used throughout. These cultures, in 16 mm diameter screw-capped tubes, were inoculated with material from patients who had received a renal allograft and were suspected of having CMV infection: either with 0.2 ml of a 1 : 1 mixture of fresh morning urine and M 199 containing 2% fetal bovine serum (purchased from the Flow Laboratories, alleged to be free from mycoplasma contamination), or with a 5% w/v suspension of lung tissue, obtained at postmortem examination. The culture tubes, four for each specimen, were kept in a rotating drum, and fed twice weekly with 3 ml of culture medium until either a definitive cytopathic effect was noted or degeneration of cells occurred. The cells were then fixed in 2% cacodylate-buffered glutaraldehyde (pH 7.4), scraped off the glass, and post-fixed with 1% collidine-buffered osmium tetroxide. These cells were then dehydrated and embedded in EPON 812. Ultra-thin sections were examined either in a RCA EMU 3G or in a Philips 300 electron microscope. Of the four CMV strains examined, three were isolated from urine (VL 2062 U, 2072 U and 2090 U) and one from lungs (VL 2080 L).

Results and discussion

The ultrastructural appearance of CMV infection in these cells was as described by others [3-11] except for the prominent finding of plexiform vermicellar arrays in the nuclei of infected WI-38 cells in three out of the four isolates examined (VL 2062 U, 2072 U and 2080 L). These were usually multiple, and were found in strikingly close proximity to areas of virus nucleocapsid formation in the electron-dense area of the nucleus (Figs 1, 2), although not

Table I
Quantitative relationship of PVA's to CMV fabrication in WI-38 cells

CMV Isolate	≠ of CMV-infected cells		≠ of normal cells	Total
	with PVA's	without PAV's		
VL 2062 U*	5	18	68	91
VL 2077 U	1	10	38	49
VL 2080 L**	5	12	44	61
VL 2090 U	0	26	68	94

* Isolated from urine

** Isolated from lung

exclusively so (Fig. 3). The structures were always globular, well delineated from the adjacent nucleoplasm, and consisting of intertwined strands varying in diameter, measuring on the average 45 nm in diameter, in close agreement with the size of less than 500 Å given by SWANSON *et al.* for PVA's in HVH-infected neurons. CMV virus nucleocapsids were either intermixed with the electron-dense vermicellar arrays, or were in close proximity to them with virus being fabricated in the dense nucleoplasm around the arrays. The PVA's were always seen in nuclei of CMV-infected WI-38 cells, but not all the virus-infected cells showed the presence of these bodies. Table I shows the results of the quantitative study of the relationship of PVA's to CMV fabrication in WI-38 cells. In the case of VL 2090 U isolate, no PVA's were seen in any of the 26 CMV-infected cells examined; in the case of the 2077 U isolate, only one out of ten CMV-infected cells examined contained PVA's, while in the case of isolates VL 2062 U and 2080 L, 5 out of 23 and 5 out of 17 respectively, *i.e.*, only a fraction of CMV-infected cells, showed the presence of PVA's.

As such PVA's were found in a small percentage of infected cells, their association with CMV fabrication in the WI-38 cells may be fortuitous, or it might perhaps, though this is unlikely, be a CMV strain-related characteristic of primary isolates in this cell line. As neither PATRIZI *et al.* [7] nor KANICH and CRAIGHEAD [10, 11] noted the presence of these structures in WI-38 cells

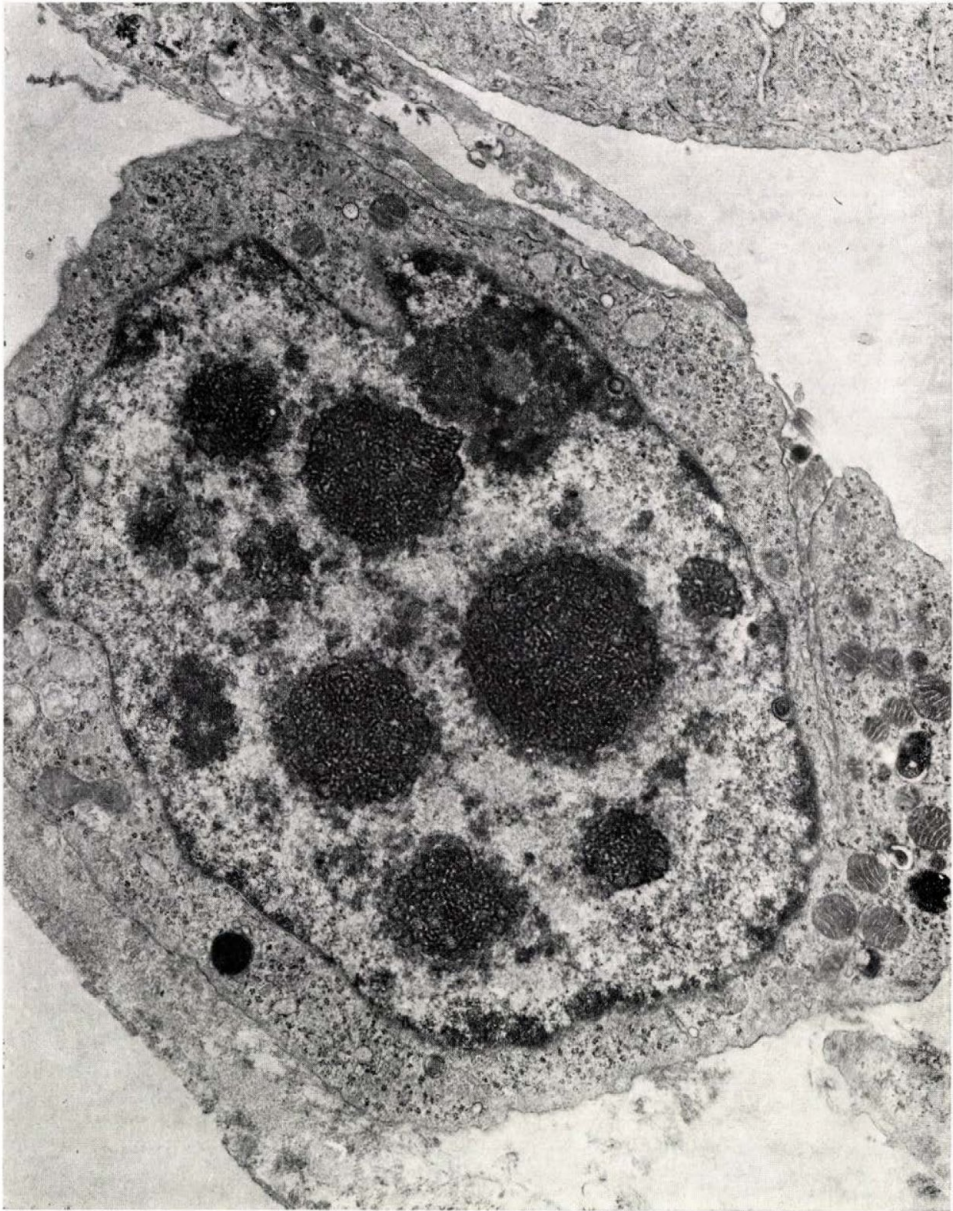


Fig. 1. Plexiform vermicellar arrays in the nuclei of CMV-infected WI-38 cells (isolate VL 2062 U); approximate magnification, $\times 7000$

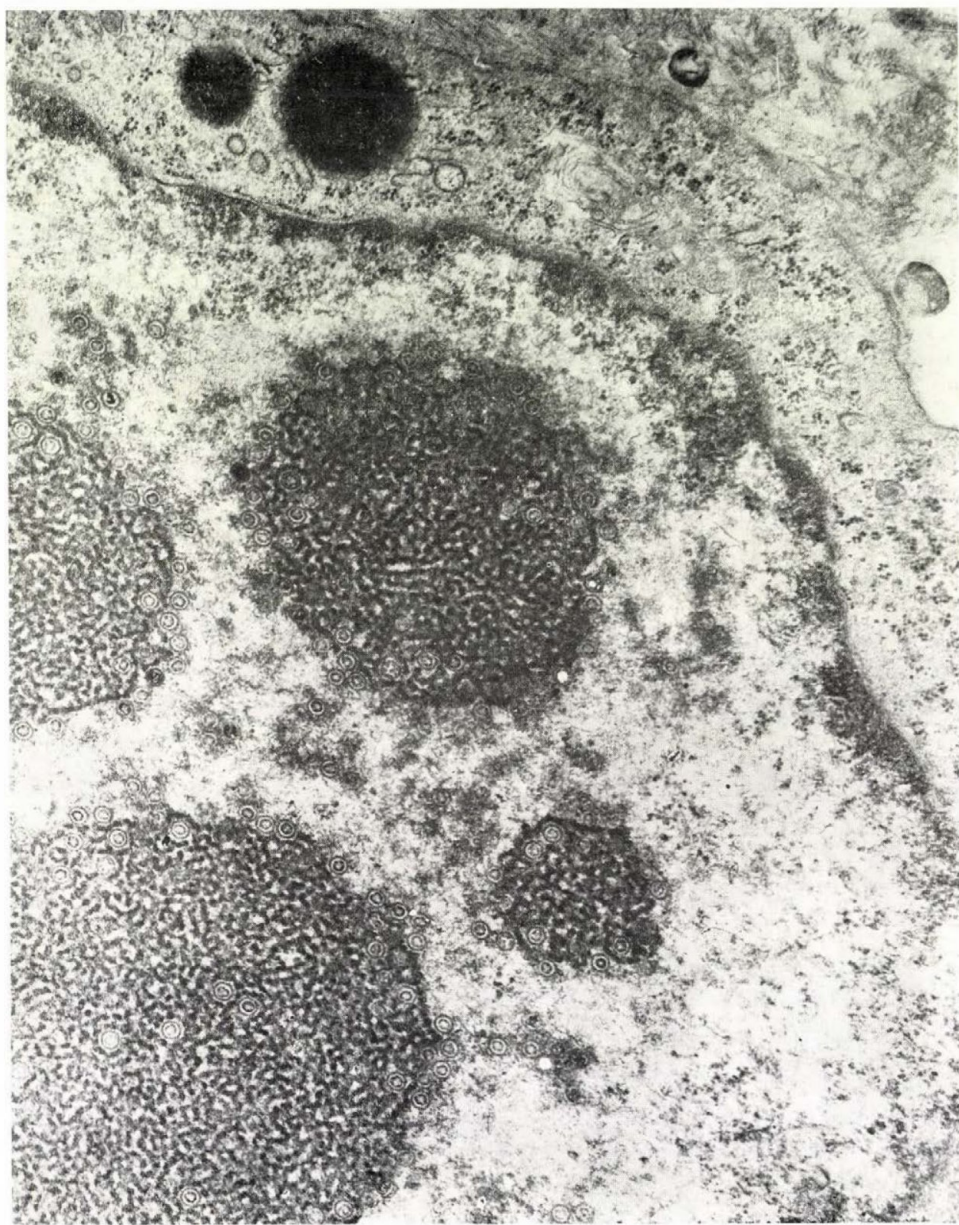


Fig. 2. Higher magnification of CMV-infected WI-38 cell nuclei showing the intimate relationship of CMV nucleocapsids to the plexiform vermicellar arrays (isolate VL 2062 U); approximate magnification, $\times 13,800$

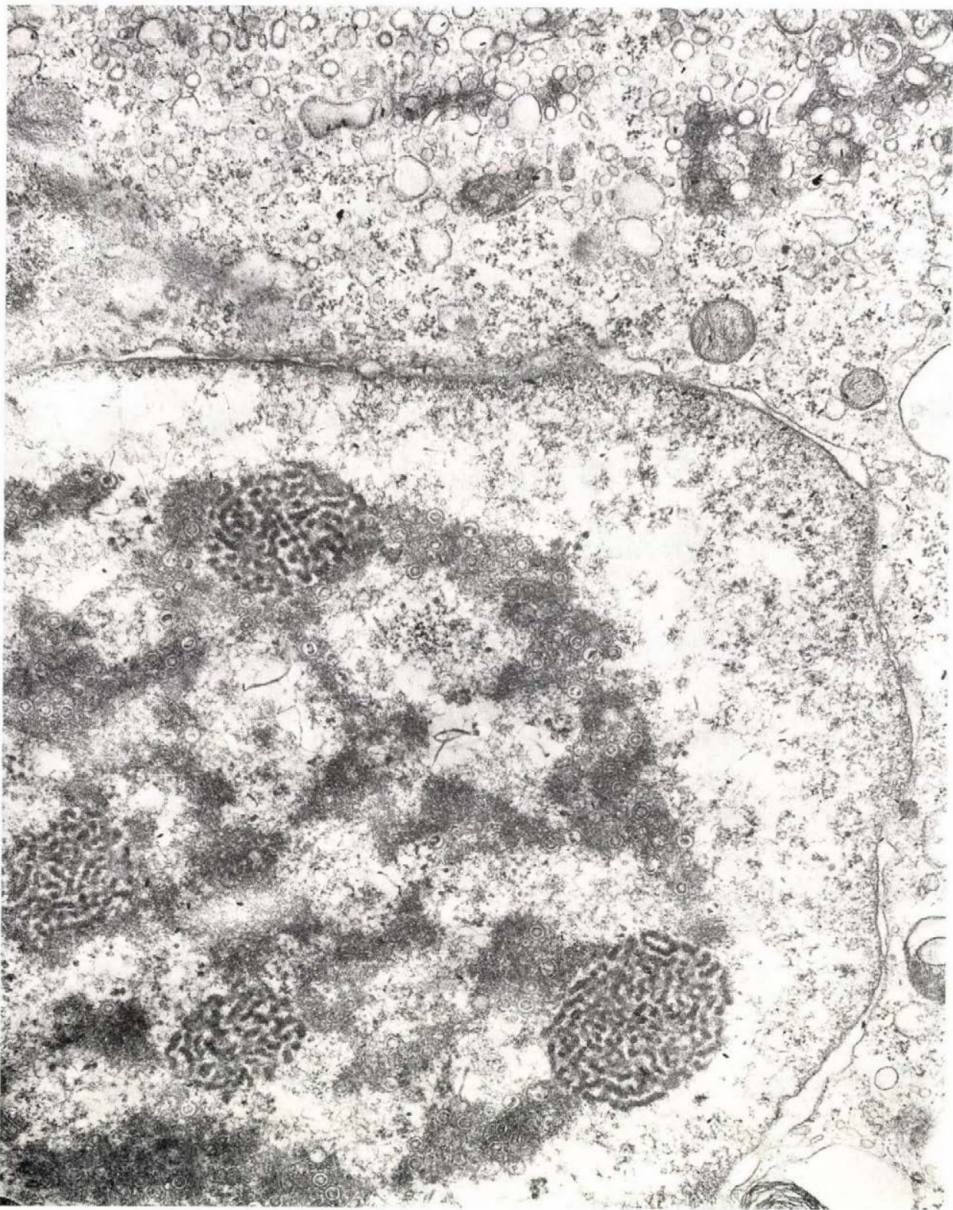


Fig. 3. CMV-infected WI-38 cell, showing plexiform vermicellar arrays adjacent to CMV-nucleocapsid formation (isolate VL 2080 L); approximate magnification, $\times 13,200$

infected with primary isolates of CMV, and in view of the small percentage of cells showing the presence of these PVA's, a fortuitous relationship is the more likely explanation.

No information is available about the nature or origin of these structures. They were never seen in non-CMV-infected WI-38 cells. There was no apparent relationship of these structures to the nuclear membrane, as they were always seen inside the nucleus, nor to the nucleolus, as this was always well-preserved in the early stages of infection, usually located near to the nuclear membrane (Fig. 1) and distinctly separated from these structures. The PVA's, however, were always located in close proximity to the electrondense granular material of the intranuclear inclusion, from which they may form by some so far unexplained mechanism.

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TOPOLOGIC ANALYSIS OF THE FATE OF TRANSFECTING SP50 DNA IN COMPETENT CELLS OF BACILLUS SUBTILIS

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Summary. A method allowing a separate study of infection and transfection has been elaborated for examining the fate of infective SP50 DNA. Under optimum conditions of transfection, successful infection with SP50 DNA of *Bacillus subtilis* 168 Indole⁻ competent cells is a "multipowered dose response" process. Infection with phage SP50 did not occur in the transfection milieu, while optimum conditions for infection were unsuitable for transfection. In the transfection milieu only one plaque forming centre (p.f.c.) was produced in each successfully infected competent cell, and there was no phage reproduction. In cells transferred into a milieu ensuring infection, phage reproduction took place and ended with a burst size of 200. In the transfection milieu, p.f.c. production could be neutralized with SP50 antibody after the 60th min. In transfected cells made incompetent in infection milieu the p.f.c. reproduced and were neutralized only after the end of the lysis. Protoplasts were unsuitable for infection and transfection. In protoplasted transfected cells, p.f.c. were not further synthesized. In transfected cells suspension, from the 75th min onward, the majority of p.f.c. was present extracellularly; the number of p.f.c. in washed cells was lower by one exponent. The number of p.f.c. in protoplasts was very low. Cell proteins labelled with [¹⁴C]tryptophan were absent from the trichloroacetic acid precipitate of the filtrate of transfected cells, while the cells showed radioactivity. P.f.c. were produced in transfection without the lysis of infected cells.

Transfection is a suitable means for studying the genetics and molecular biology of virus reproduction [1–4]. The use of isolated and purified nucleic acid allows to investigate the details of virus synthesis, DNA adsorption, replication and initiation, thus the fate within the cell of external nucleic acid [5–9].

In previous experiments [10, 11], the optimum conditions for transfection were established and a method was worked out allowing the separate examination of infection and transfection. We investigated the kinetics and mechanism of transfection under conditions excluding the possibility of infection and of a further reproduction of the virion produced in the course of transfection [11]. It was found that in transfection phage DNA replication took place on the cell surface (cell membrane, mesosomes). The present experiments have offered some further data concerning the fate of infective DNA in the cell.

Materials and methods

Bacterial strains. *Bacillus subtilis* 168 Wild and Indole⁻ auxotrophic strains were obtained from the collection of Professor W. SZYBALSKI, McArdle Laboratory, Madison, Wisconsin (U. S. A.).

Phage strain and DNA. Phage SP50 [12, 13] was used. DNA was prepared as described in [9].

Immune serum was prepared as previously [11]. It was purified by fractionating the immune globulins [14], dialyzed against saline and stored in the deep freeze; $K = 1400$.

Culture media were described in [11].

Other reagents. Desoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J.) stock solution, 1 mg/ml; lysozyme (Koch-Light Labs. Ltd., Colnbrook) stock solution, 10 mg/ml; sucrose (BDH Chemicals Ltd., Poole) M-III solution, 1 M; albumin, bovine fraction V., B grade (Calbiochem, Los Angeles, Ca.) stock solution, 100 mg/ml; [^{14}C]DL-tryptophan (unknown specific activity), 4 mg/ml, 4×10^6 cpm; trichloroacetic acid (Reanal, Budapest), 10% and 5% solutions; scintillation fluid: P.P.O. and P.O.P.O.P. (Nuclear Enterprises Ltd., Sighthill, Edinburgh) 5% and 0.5% dissolved in toluol; membrane filter HAWG 025 00, HA 0.45 μ , 25 mm (Millipore Filter Corp., Bedford).

Transfection. Competent cells were prepared and transfected as described previously [11]. Some modifications were as follows. SP50 DNA stock solution (250 $\mu\text{g}/\text{ml}$) was diluted with medium M-III. For lysozyme treatment and preparation of protoplasts undiluted competent cells (3×10^8 cells per ml M-II) were transfected. For a quantitative determination of plaque forming centres (p.f.c.) the samples were diluted with medium M-III and adequate dilutions were layered over T-agar plates. Incompetent cells of *B. subtilis* 168 Indole⁻ were used as indicator.

For transfection with protoplasts, SP50 DNA was diluted with medium M-III containing 0.5 M sucrose. In parallel experiments 1% albumin was added to the transfection medium [15, 16].

Immune serum treatment. Neutralization of p.f.c. was made as previously [11], with the modification that to every sample diluted in medium M-III, purified immune globulin fraction was added at 1 : 100 dilution.

Lysis of bacterial cells with lysozyme. Transfected cell suspensions were sampled at intervals and the undiluted or 10^{-1} diluted samples were treated in M-III containing 10^{-2} M NaN_3 with 100 μg lysozyme per ml at 37°C for 20 min. The number of p.f.c. was determined as described above.

Preparation of protoplasts from competent cells and from cells transfected with SP50 DNA was performed as follows. After adding 10^{-2} M NaN_3 , the samples were centrifuged at 4°C and the pellet was suspended in M-III containing 10^{-2} M NaN_3 and 0.5 M sucrose. Lysozyme was added at 100 $\mu\text{g}/\text{ml}$, then the cell suspension was incubated at 37°C for 20 min. The suspension was checked under a phase-contrast microscope and the number of survivors, which decreased to under 0.02% on the average, was determined by streaking out different dilutions. The protoplast suspension was centrifuged, washed and resuspended in the same volume of M-III containing 0.5 M sucrose. The number of p.f.c. was determined in the bacterial cells, protoplasts and in their supernatants.

Labelling of bacterial cell proteins. *B. subtilis* 168 Indole⁻ cells were labelled by culturing them at 37°C for 4 hr in medium M-I supplemented with 80 $\mu\text{g}/\text{ml}$ of [^{14}C]DL-tryptophan. Then the culture was cooled ("synchronized") at 4°C for 30 min, centrifuged and the cells were resuspended in cold M-II medium. Subsequently, the procedure described for the preparation of competent cells was performed.

The labelled competent cells were transfected with SP50 DNA and incubated for 4 hr. Two ml amounts taken at intervals were passed through membrane filter, washed with M-II and the membranes were dried. Then the membrane was placed in scintillation fluid and the radioactivity of cells retained on the membrane was measured in a Tri-Carb 2001 counter (Packard Instrument Co. Inc., Downer Grove, Ill.). The filtrate was precipitated at 4°C with 2 ml 10% trichloroacetic acid after the addition of 0.1 ml 10% albumin and left to stand at 4°C overnight. The precipitate was collected by membrane filtration and washed with 5% trichloroacetic acid. Radioactivity was measured as described above.

Results

In examining the optimum conditions of transfection we aimed at attaining that the phage produced (the p.f.c.) should not reproduce or cause secondary infection and that transfection be unsuccessful under the conditions of phage infection.

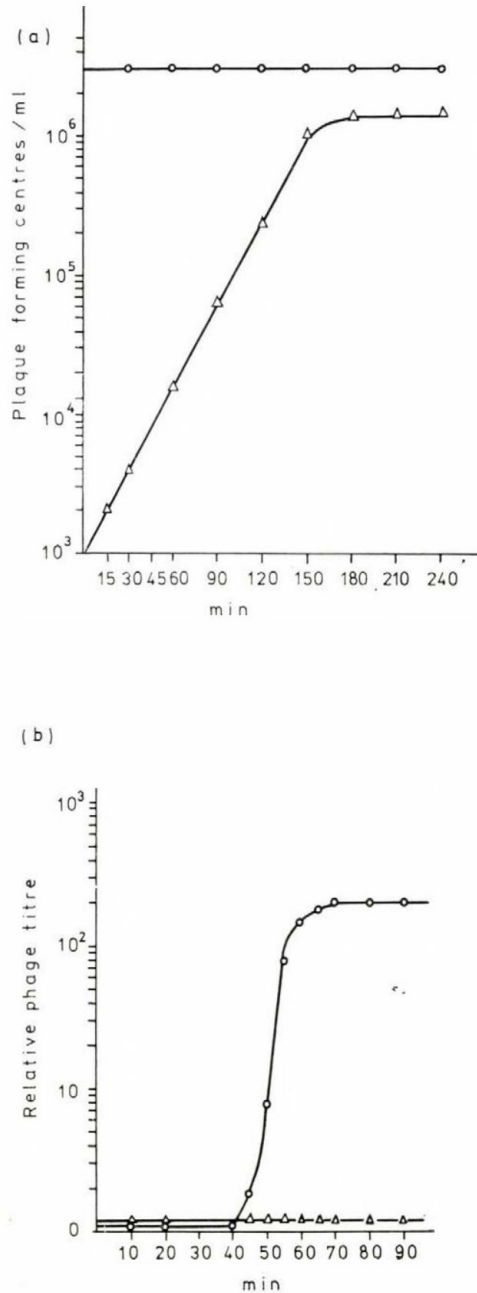


Fig. 1. Infection and transfection of competent cells of *B. subtilis* 168 Indole⁻ with page SP50 and SP50 DNA (a) in transfection medium (b) in TY medium. 1.5×10^8 cells/ml were transfected with $0.5 \mu\text{g}$ SP50 DNA/ml; 1.5×10^7 cells/ml were infected with SP50 (m.o.i. = 0.1); during incubation with aeration at 36°C , samples were taken at intervals and titrated for the number of p.f.c. Δ — Δ transfection, $\circ$ — $\circ$ infection

In the experiment presented in Fig. 1 (a), competent cells diluted in M-III were infected, as usually, with SP50 phage (infection) and with SP50 DNA (transfection). In the course of transfection the number of p.f.c. increased logarithmically and at 120 min reached the peak (1.5×10^6 p.f.c. with $0.5 \mu\text{g}$ SP50 DNA and 1.5×10^8 cells per ml). In the same milieu, phage SP50 failed to reproduce although, as shown previously [11] phage adsorption had taken place.

In the experiment shown in Fig. 1 (b), the competent cells were suspended in medium TY. For infection a one step growth curve was recorded. Transfection was examined by the usual method.

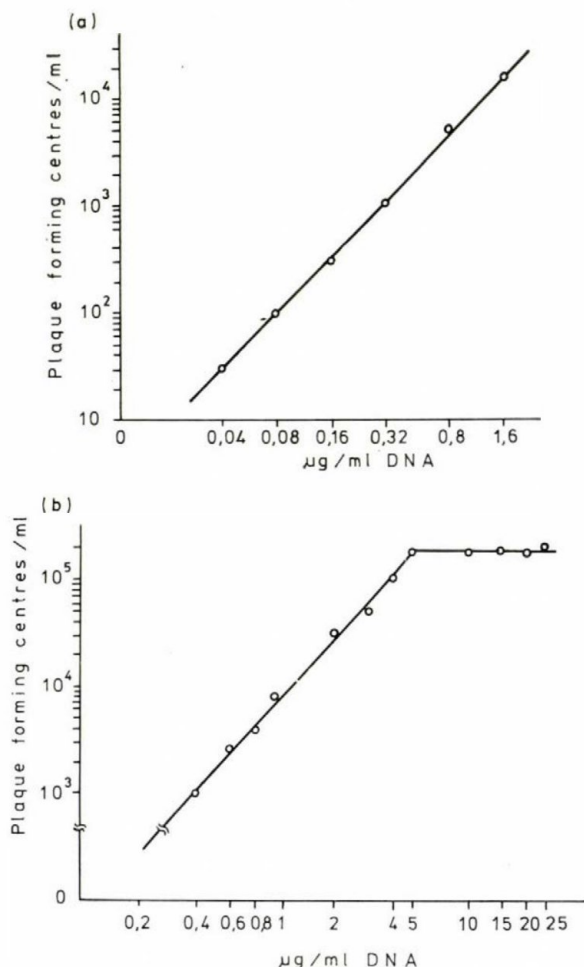


Fig. 2. Dose-response curve for the transfection of *B. subtilis* 168 Indole- with SP50 DNA. 6×10^7 cells/ml in M-II medium were mixed with equal volume of SP50 DNA diluted in M-III medium. After incubation at 36°C for 60 min, samples of the appropriate dilutions were plated in triplicate on T agar medium. (a) 0.04–1.6 µg DNA/ml; (b) 0.2–25 µg DNA/ml

The result of the one step growth curve was in agreement with our data [11] showing that for phage SP50 the burst size is 200. In the course of transfection not a single p.f.c. was produced, thus in TY medium the competent cells could not be infected. The regulating factor for the competency in the medium is the YE [17].

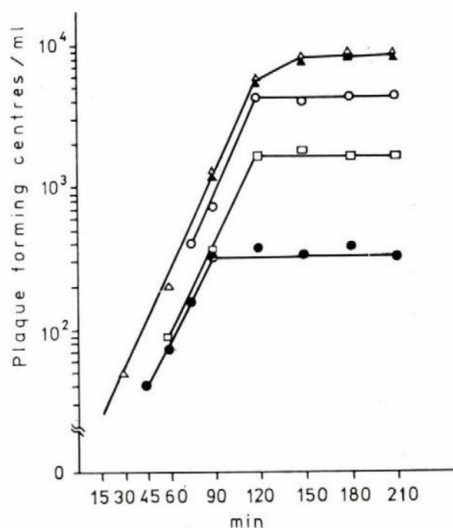


Fig. 3. DNase treatment of transfected cells. 6×10^7 cells/ml M-II were transfected with $0.1 \mu\text{g}$ DNA/ml in M-III with aeration at 36°C . 2 ml samples were taken at intervals indicated and treated with $2 \mu\text{g}$ DNase/ml for 15 min at 36°C , then the p.f.c. titre was determined, at

●—● 30 min; □—□ 45 min; ○—○ 60 min; ▲—▲ 75 min; Δ—Δ control

In addition to the milieu, the result and efficacy of transfection are influenced by the degree of competence and the concentration of DNA. The former can be controlled experimentally, but the process is less known in detail, while the latter can be well defined quantitatively for the given competent cells.

The efficacy of transfection as plotted against DNA concentration is shown in Fig. 2 (a) and (b). The curves rise logarithmically and indicate that at a given concentration a saturated state is reached when the number of p.f.c. ceases to increase. The form of the curves indicates that in SP50 DNA transfection there is a "multipowered dose response" [18].

According to the above experiments, under the conditions used the uptake of SP50 DNA was a prolonged process. This was confirmed by DNase treatment of transfected cells at different intervals after transfection (Fig. 3).

In the first 20 min of transfection, the SP50 DNA was sensitive to DNase: no p.f.c. were produced. After the 30th min there was a gradual "integration" of DNA, which became complete after 75 min, when the number of p.f.c. in

the DNase-treated sample was the same as in the control. The prolonged course of transfection may have been due to the low initial number of competent cells in the so-called competent suspension [19], and that the unadsorbed DNA was gradually used up for the infection of newly developed competent cells. In order to avoid this factor, in further experiments the infected cell suspension was treated with DNase in the 45th min of transfection.

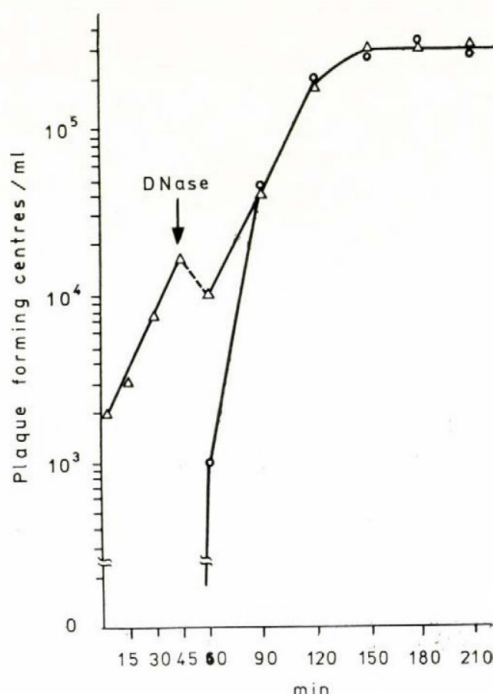


Fig. 4. Lysis of transfected cells by lysozyme. 6×10^7 cells/ml in M-II were transfected with equal volume of SP50 DNA ($1 \mu\text{g}/\text{ml}$ M-III) and incubated with aeration at 36°C . DNase treatment ($2 \mu\text{g}/\text{ml}$) was performed at 45 min. Samples were taken at intervals indicated and treated with lysozyme ($100 \mu\text{g}/\text{ml}$) at 36°C for 20 min then the p.f.c. titre was determined.

○—○ Lysozyme treatment; △—△ control

In subsequent experiments the topology of the fate of transfecting DNA and the role of structural constituents of the bacterial cell were examined by the use of lysozyme treatment.

As shown in Fig. 4, samples taken at intervals from the transfected cell suspension were lysed with lysozyme and the number of p.f.c. was determined. It is striking that in the lysates the p.f.c. appeared only after 60 min. They corresponded in number (10^3) to the number of cells infected at 0 min, that is, to the number of actually competent cells. At 90 min the number of p.f.c. in the test suspension had reached the control value, to persist at the same

throughout the experiment. These data indicate that in one cell only one phage particle is produced (burst size 1). Accordingly, the "phage" is produced in the infected cell, but it is not reproduced; see also the data in Fig. 1 (a).

If, however, the infected cells are treated with DNase at 45 min and then at 60 min are transferred into TY medium, the reproduction of p.f.c.

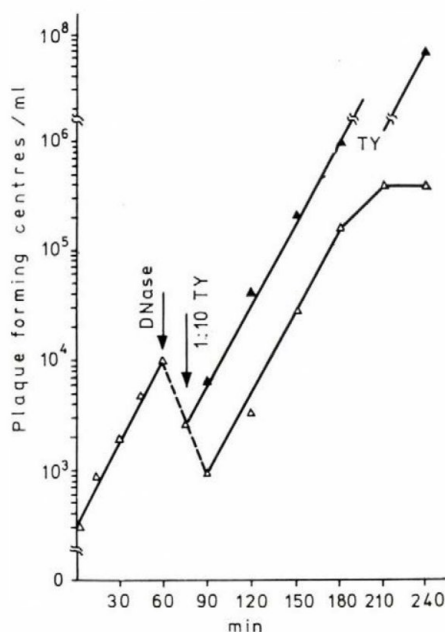


Fig. 5. Reproduction of SP50 phage in TY medium after transfection. 6×10^7 cells/ml in M-II were transfected with equal volume of SP50 DNA ($1 \mu\text{g}/\text{ml}$ M-III) and incubated at 36°C with aeration. DNase treatment ($2 \mu\text{g}/\text{ml}$) was performed at 37°C for 60 min. Then the sample was diluted 1 : 10 in TY medium and the number of p.f.c. was titrated at intervals indicated.

▲—▲ TY medium; △—△ control

present in the cell begins, and at 240 min the values correspond to those shown in Fig. 1 (b), where the burst size is 200.

Antibody treatment of samples shown in Fig. 5 gave interesting results. After neutralization with purified antibody, the number of p.f.c. was determined (Fig. 6). It was striking that in the transfection milieu the p.f.c. were neutralized after 60 min and in subsequent samples their number decreased to nil. At the same time the number of p.f.c. in cells transferred into TY medium increased gradually. Accordingly, in transfection milieu, after a relatively short "vegetative phase", the p.f.c. were accessible to antibodies, while during the reproduction taking place in TY medium, no neutralization was observed.

In view of our previous results we assumed that in the course of transfection with SP50 DNA, p.f.c. synthesis took place on the cell surface [11].

As this assumption was not in agreement with the general conception of phage reproduction, for confirmation the following experiments were performed.

If in transfected cells p.f.c. synthesis occurs in the cytoplasm, phage synthesis in protoplasted bacteria should be unaltered after a certain period.

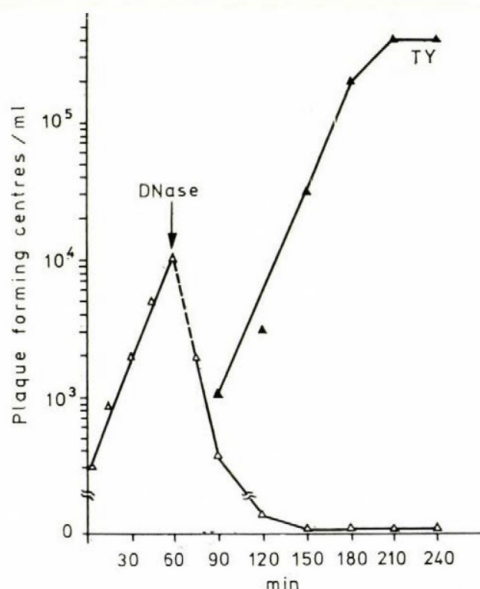


Fig. 6. Neutralization of p.f.c. with purified SP50 antibody. 6×10^7 cells/ml M-II were transfected with equal volume of SP50 DNA ($1 \mu\text{g}/\text{ml}$ M-III) and incubated at 36°C with aeration. DNase treatment ($2 \mu\text{g}/\text{ml}$) was performed at 60 min. The sample was diluted 1 : 10 in TY medium, incubated as before and sampled at the indicated intervals. Appropriate dilutions of the samples in M-III medium were treated with 1/10 vol antibody diluted 1 : 100. After incubation for 10 min at 36°C the samples were titrated for p.f.c. number. $\blacktriangle$ — $\blacktriangle$ TY medium; $\triangle$ — $\triangle$ control

This assumption was based on experiments with *Escherichia coli* protoplasts and phages [15, 16, 20, 21].

Competent cells of *B. subtilis* 168 Indole⁻ were infected with SP50 DNA in the usual manner, treated with DNase at the 45th min and at the 75th min the bacteria were protoplasted and the suspension was incubated further.

As shown in Fig. 7, after protoplasting the synthesis of p.f.c. ceased and during further incubation their number remained at a given level. Thus, the cell wall, unlike in *E. coli* spheroplasts, is an essential structural factor in p.f.c. synthesis in transfected cells.

According to our other experiments, protoplasts prepared from competent and incompetent cells of *B. subtilis* 168 Indole⁻ strain could not be transfected or infected with phages either under the present conditions or in other experiments [15, 16].

In DNA replication, initiation and other biosynthetic processes the cell membrane plays a prominent role [22-30]. This has been considered when studying other cell fractions for the elucidation of the topology of transfection.

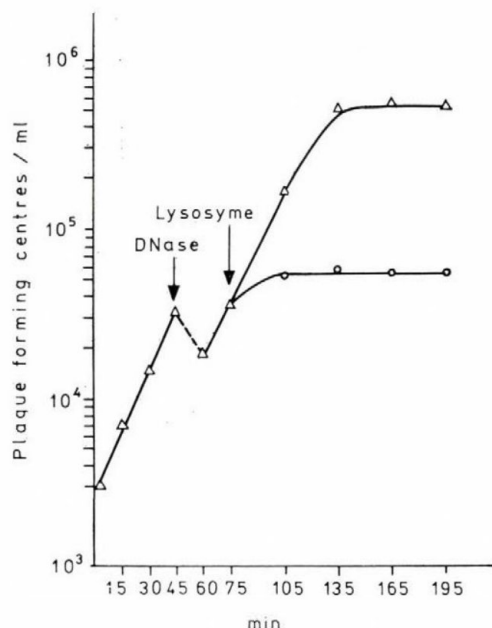


Fig. 7. Protoplasting of transfected cells. 3×10^8 cells/ml M-II were transfected with equal volume of SP50 DNA ($1 \mu\text{g/ml}$ M-III with 1 M sucrose). Incubation was performed at 36°C with aeration. DNase treatment ($2 \mu\text{g/ml}$) was carried out at 45 min. A sample was taken for protoplasting at 75 min and treated with lysozyme ($100 \mu\text{g/ml}$). The number of p.f.c. was determined at intervals indicated. $\circ$ — $\circ$ Protoplasts; $\triangle$ — $\triangle$ control

Fig. 8 shows that the number of p.f.c. increased up to the 120th min logarithmically and reached a peak of 5×10^5 at the given cell count and SP50 DNA concentration. In the supernatant obtained after centrifugation, the number of p.f.c. was considerable at 75 min and from the 90th min onward it nearly reached the value found in the unwashed cell suspension (4×10^5). In the washed cell suspension the number of p.f.c. was lower by one exponent. After protoplasting this cell suspension, the number of p.f.c. decreased by 3 exponents. The whole difference in p.f.c. number was present in the lysozyme lysate.

In view of this the question arose whether the p.f.c. present in the supernatant of the transfected suspension are particles liberated after the disintegration of cells as in the course of virus reproduction, or are released without a lysis of the infected cells. To answer this question, cytoplasmic proteins were

labelled, samples were taken at intervals from 0 to 240 min after transfection, then membrane filtered and measured for radioactivity both in the cells and in the filtrate.

In trichloroacetic acid-precipitable substances of the filtrate, no activity was present. The radioactivity of the cells remained unaltered throughout

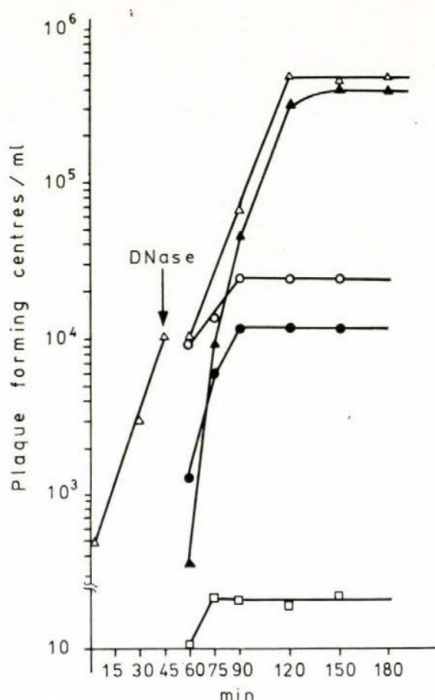


Fig. 8. Fractions of transfected and protoplasted cells. 6×10^8 cells/ml M-II were transfected with equal volume of SP50 DNA ($1 \mu\text{g}/\text{ml}$ M-III with 1 M sucrose). Incubation was performed at 36°C with aeration. After DNase treatment ($2 \mu\text{g}/\text{ml}$) at 45 min a sample was taken for protoplasting with lysozyme ($100 \mu\text{g}/\text{ml}$). After incubation as before samples were taken for fractionation and titration of p.f.c. ▲—▲ Supernatant of the centrifuged whole culture; Δ—Δ whole culture; ●—● supernatant of the centrifuged protoplasts; ○—○ pelleted, washed and resuspended cells; □—□ pelleted, washed and resuspended protoplasts

the experiment, indicating that the release of p.f.c. was not associated with cell disintegration.

Discussion

Successful transfection depends on a number of factors the total of which is termed competence. Theories developed in connection with this problem have been reviewed by SPIZIZEN *et al.* [31]. According to HOTCHKISS [32] competence expresses an unstable ratio between virus (p.f.c.) synthesis and specific phase of bacterial growth, special cellular functional stage or biosynthetic processes.

This ratio becomes advantageous for the cell if the ingredients and quality of the medium promote cell multiplication (the cells are incompetent). Under restricted nutritional conditions the cells become competent. As competence is controlled by certain genes [31], these considerations hold true only for a given, genetically well-characterized bacterial strain.

Competence can be increased in various ways, for example by UV irradiation [33]. In our experiments, high concentrations of inorganic salts, especially of Mg^{++} , yielded good results [10]. This finding is assumed to be due to the fact that at 10^{-2} M $MgSO_4$, the nascent messenger RNA is bound to the polysome within the DNA membrane complex [34].

Competence of the host cell is not a sole factor of the efficacy of transfection. Recombination of different degrees depending on the molecular structure of DNA, also plays a part [18]. This is indicated by the shape of the dose-response curve, which in the case of SP50 DNA is a "multi-powered" one; see Fig. 2. (a) and (b). In the opinion of SPATZ and TRAUTNER [4], in transfection two kinds of recombination may occur: "primary recombination" takes place obligatorily prior to DNA replication, while "secondary recombination" occurs between vegetative phages. Thus, in SP50 DNA transfection, the co-operation of at least three DNA molecules is needed for primary recombination.

Our results are in accordance with the above considerations, secondary recombination could be excluded as there was no p.f.c. reproduction (Fig. 4).

As to the fate of infective DNA there are several problems to solve: its adsorption, uptake, intracellular state, replication of DNA, initiation of replication and virus reproduction. In investigations into these processes, the main interest has been focussed upon the structural and functional state of the cell wall and cell membrane.

The adsorption and penetration of the virus is known in detail. Yet, further studies of plasmolysed *E. coli* cells and of the isolated "envelope-membrane complex" yielded interesting data. Phage adsorption seems to occur on 200–400 sites where the cell wall and the membrane are attached [27]. An isolated "envelope-membrane complex" adsorbs the phage and DNA injection occurs, but separately neither the envelope nor the membrane is able to function in this manner. Concerning adsorption and penetration, it is irrelevant whether the membrane of the complex has been prepared from phage sensitive or phage resistant strains, but the envelope must originate from phage sensitive organisms [35].

Our results with *B. subtilis* protoplasts are in agreement with the above data in that protoplasts devoid of cell wall cannot be infected with phage or with phage DNA. Successful transfection with protoplasts of Gram-negative bacteria [15, 16, 20, 21] is attributed to the presence of cell wall debris remaining on the cell membrane.

The regular segregation of chromosomes occurs by the attachment to the cell membrane of one site of the chromosome. This assumption, advanced by JACOB *et al.* [36] on the basis of genetic experiments and morphological observations, has been confirmed by electron microscopic studies [37]. The invaginated part of the membrane, where the chromosome is attached, was termed mesosome. Other experiments showed that the so-called "fast sedimenting fraction" of lysozyme-treated *B. subtilis* is actually a small part of the cell DNA bound to the membrane [38, 39]. The pulse labelled DNA bound to the membrane has been termed replicon or replication origin.

For the binding of circular DNA to the membrane an enzyme complex is needed [40, 24]. Concerning the properties of mesosomes [41] and the stages of membrane and nucleus association [42], several data are available.

Replication of phage DNA, too, takes place on the membrane. Studies of T4 [43], T7 [44], lambda [30], M13 [45, 46] and ϕ X174 [28] phages confirmed that the "parented DNA" is associated with the "rapidly sedimenting host cell component". A further analysis of fractionated and extracted parental DNA showed that at least half of the membrane-bound parental DNA was incorporated into the "replicative intermediate" with a virus chain more than one unit in length [45].

Under the conditions applied in our experiments the transfected cells failed to produce p.f.c. after protoplasting. If for DNA replication only the cell membrane would be needed, the number of p.f.c. ought to increase further. Accordingly, in *B. subtilis* the cell wall is an obligatory factor of phage DNA replication. It may be assumed that the enzyme necessary for DNA replication was removed with the disintegration of the cell wall. Enzymes of DNA synthesized *in vitro* are near to the replication origin [23] which, in our experiments, was located probably on the outer surface of the membrane (see Fig. 8). From the 60th min onward, the p.f.c. appeared in the medium and after the 90th min, more than 90% were released but not filterable until the 120th min [47]. After removing the cell wall, the number of p.f.c. remaining in the protoplasts was very low. It may be assumed that the replication of SP50 DNA takes place between the cell wall and the membrane and both structural elements are necessary for p.f.c. production. Their particular topological situation may be responsible for the fact that p.f.c. failed to reproduce.

The role of "membranous bodies" in DNA recombination and replication is indicated also by other data. Electron microscopic autoradiography with *B. subtilis* [25] showed that the donor [3 H]DNA remained outside the membranous bodies, while molecules derived from the adsorbed DNA were closely bound to them.

In our experiments, SP50 DNA recombination and replication were not followed by reproduction. If the transfected cells were transferred into TY medium not earlier than 30 min after transfection, the cells became incompet-

ent and reproduced filterable phages from the p.f.c. If the transfected cells were made incompetent in the above manner sooner than 30 min, not even p.f.c. were produced. For this regulatory mechanism a dialysable component of the yeast extract seemed to be responsible, which derepresses the restriction enzyme of DNA [15, 17].

The above data have been confirmed by the SP50 antibody treatment of transfected cells and the labelling of cell proteins with [^{14}C]tryptophan. P.f.c. produced after DNA recombination and replication can be neutralized in the transfected milieu, thus they correspond to protein-coated free inactive particles (Fig. 8). P.f.c. reproducing in TY medium cannot be neutralized and are accessible to antibodies only after a total lysis.

There is no visible and measurable sign of lysis in the transfection milieu; labelled proteins cannot be detected in the trichloroacetic acid precipitate of the filtrate.

On the basis of these findings it may be assumed that recombination and replication of the infective DNA takes place on the outer surface of the cell membrane. P.f.c. are released without a lysis of the cells. Their reproduction occurred only in cells made incompetent after recombination or replication of the infective DNA had taken place in competent cells.

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PIRAZOCILLIN: A NEW ACID AND PENICILLINASE RESISTANT SEMISYNTHETIC PENICILLIN

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Summary. The chemotherapeutic properties of Pirazocillin, a new pyrazole-carbonic acid containing semisynthetic penicillin were tested and compared with that of dicloxacillin. The compound at concentrations of 0.1–0.2 $\mu\text{g/ml}$ possesses an *in vitro* bacteriostatic activity on Gram-positive microorganisms including penicillin sensitive and resistant *Staphylococcus aureus* strains. Pirazocillin activity depended on pH of the medium, the optimum was at pH 7.0. At 1.0 $\mu\text{g/ml}$ concentration it exhibited a bactericidal effect but only on multiplying microorganisms. Pirazocillin-resistant cells were unfrequent, and the sensitivity of cells cultivated in the presence of Pirazocillin decreased slowly, to about 25% after 15 transfers. Pirazocillin was stable in aqueous solution; in acid medium (pH 2.0) it was found to retain 70% of its original activity after 6 hr, and in artificial gastric juice 20% after 3 hr. From a 10 $\mu\text{g/ml}$ solution, 88% was bound to serum proteins. Under the given experimental conditions 50 IU/ml penicillinase decreased the potency of Pirazocillin by 10% in 2 hr, while enzyme activity dropped to 14% of its original level. The DC_{50} value for mice infected with penicillin-sensitive *S. aureus* Smith strain was, intravenously 16; subcutaneously 5 and orally 26 mg/kg; whereas in mice infected with penicillin-resistant *S. aureus* 1115 strain, intraperitoneally 35; and orally 38 mg/kg. Blood levels were estimated in mice and dogs. The results of comparative experiments run simultaneously with dicloxacillin, demonstrated that Pirazocillin was more stable and was bound to proteins to a lesser degree but its absorption from the stomach of mice was weaker as compared with dicloxacillin.

Pirazocillin, 1-(2,6-dichlorophenyl)-4-methyl-5-pyrazolyl-penicillin-Na, is a semisynthetic derivative of 6-amino-penicillanic acid (6-APA). The compound was selected from among the derivatives of 6-APA acylated by pyrazole carbonic acids in the course of a programme aimed at selecting non-toxic penicillin derivatives of great stability, thus for oral administration and ensuring a potent strong effect against penicillin-resistant staphylococci. In the course of the studies, many compounds exhibiting similar properties were synthesized among which dicloxacillin gained the most extensive practical employment. This paper reports on results obtained with Pirazocillin.

Materials and methods

Compounds. Pirazocillin was synthesized and supplied by FEHÉR *et al.* [1]. The structural formula is shown in Fig. 1 and that of dicloxacillin in Fig. 2. Comparative data are collected in Table I. Pirazocillin is a colourless, odourless, hygroscopic powder, well soluble in water and insoluble in ethanol. Specific rotation: $[\alpha]_{\text{D}}^{25} = 202^\circ$ ($C = 1$, H_2O). The pH of its aqueous solution is 5.8–6.5. The compound can be characterized on the basis of its specific IR spectrum and values obtained by thin-layer chromatography. The compound used throughout the experiments was homogeneous and contained at least 98% of active substance. The solutions, if not indicated otherwise, were made with distilled water. The compounds employed in the

course of comparative studies were: penicillin-G (Biogál Pharmaceutical Plant, Debrecen, Hungary), oxacillin, 5-methyl-3-phenyl-4-isoxazoly-penicillin-Na (Stapenor, Bayer), cloxacillin, 5-methyl-3-o-chlorophenyl-4-isoxazoly-penicillin-Na, Orbenin (Beecham Research Laboratory), dicloxacillin, 5-methyl-3-(2,6-dichloro-phenyl)-4-isoxazoly-penicillin-Na, Dichlor-Stapenor (Bayer), methicillin, 2,6-dimethoxy-phenyl-penicillin-Na, Celbenin (Beecham Research Laboratory). Penicillinase enzyme was prepared in our laboratory [2].

Pirazocillin

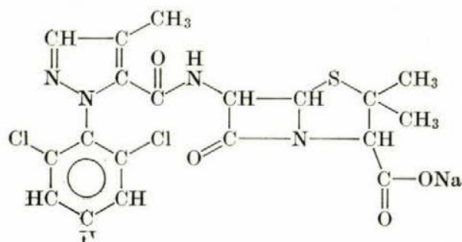


Fig. 1. Pirazocillin: 1-(2,6-dichlorophenyl)-4-methyl-5-pyrazolyl-penicillin-Na

Dicloxacillin

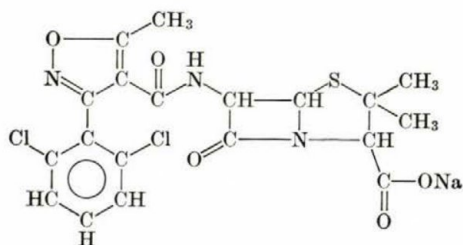


Fig. 2. Dicloxacillin: 5-methyl-3-(2,6-dichloro-phenyl)-4-isoxazoly-penicillin-Na

Table I

Dicloxacillin and Pirazocillin

Name	Structure	IU/mg 1000 IU/mg	Formula Molecular weight
Dicloxacillin		1164 0.859	$C_{19}H_{16}Cl_2N_3NaO_5S \cdot H_2O$ mol wt 510 · 34
Pirazocillin		1169 0.856	$C_{19}H_{17}Cl_2N_4NaO_4S \cdot H_2O$ mol wt 509 · 19

Measurement of antibacterial effect. Minimum inhibitory concentration (MIC) was assayed by employing a twofold serial dilution [3] using Brain Heart Infusion CM 225 (Oxoid, London) medium with various supplementations as indicated. For the measurement of antimycobacterial effect the medium described by DUBOS and MIDDLEBROOK [4], and for comparison, Kirchner-Sauton's medium [5] was used. Concentration of the 16-hr-old inoculum used for the determinations varied between 1 to 5×10^5 cells/ml. Turbidity of cell suspensions was measured photometrically (Spectromom 502, Hungarian Optical Works, Budapest, Hungary) [3].

Measurement of bactericidal effect. The relative rates at which the various compounds of the same concentration killed cells used as test organism were determined. A 6–8 hr Brain Heart Infusion CM 225 culture in the log phase, and a 30 hr accordingly diluted culture in the stationary phase, were exposed at 37°C to the effect of various concentrations of each antibiotic. The viable cells remaining in the cultures were separated by membrane filtration [3] at different intervals and after 18 hr cultivation at 37°C determination was performed in a medium containing 1.5% agar. In Fig. 5 average values are indicated. All samples and their dilutions were plated out in parallel on 2% CM 225 Oxoid agar by spreading 0.1 ml over the agar surface. After incubation overnight at 37°C, the number of colony-formers was determined.

Agar diffusion test was performed as described in [6]. Composition of the agar plate was: peptone, 6.0 g; enzymatically digested casein, 4.0 g; yeast extract, 3.0 g; meat extract, 1.5 g; glucose, 1.0 g; agar, 15.0 g; made up with water to 1000 g and adjusted to pH 6.8. As indicator organisms, *Staphylococcus aureus* ATCC 6538 P and *S. aureus* 53 (penicillin-resistant) strains were used; 16–18 hr cultures were mixed with the medium cooled to 45–48°C. In the case of *Bacillus subtilis* ATCC 6633, the spore suspension [7] was mixed to the medium.

Development of drug-resistance. In order to obtain data regarding the frequency of drug-resistant cells, *S. aureus* 53 strain producing β -lactamase was cultured in liquid medium at 37°C for 18 hr. From this culture, 0.5 ml was transferred into 4.5 ml volumes of media containing antibiotic at geometrically increasing concentrations. The procedure was repeated daily by using just viable cells as inoculum. The MIC values obtained daily are expressed as a function of the number of transfers.

For analyzing the resistant variants, the replica plate method described by LEDERBERG and LEDERBERG [8] was used. The diameter of the velvet disk was 8 cm.

Acid stability estimations. Pirazocillin and isoxazoly-penicillin derivatives at 200 μ g/ml were added to glycine buffer (pH 2.0), kept at 23°C and their activity was determined at intervals. Bioassay was accomplished after neutralization in 1/15 M phosphate buffer (pH 7.0) by the agar-diffusion technique.

A similar procedure was carried out when the stability of compounds dissolved in artificial gastric juice at a concentration of 700 μ g/ml was studied. The artificial gastric juice [9] consisted of NaCl, 2.0 g; pepsin, 3.2 g; 25 g 10% HCl solution made up to 1000 g with water, pH 1.3.

Binding of Pirazocillin to serum proteins. The various antibiotics differ in the extent of their binding to serum proteins. Separation of antibiotics free or bound to serum proteins was performed by dialysis according to SCHOLTAN and SCHMID [10] and the antibiotic content of the solution was estimated by the agar-diffusion test with the use of "bovine serum albumin fraction V" (Oxoid) and freshly prepared bovine and human sera.

Measurement of stability of semisynthetic penicillins against penicillinase. For this purpose *Bacillus cereus* 569 H penicillinase was used after adequate purification [2]. Substances were added to a penicillinase solution of 50 IU/ml concentration to allow a final concentration of 5×10^{-6} M/ml (= 2200 μ g/ml of oxacillin, 1940 μ g/ml methicillin and 2540 μ g/ml of Pirazocillin and dicloxacillin), then the solutions were incubated at 30°C. At intervals samples were taken and their penicillanic acid concentration was determined iodometrically [2].

Investigation of the effect on penicillinase of Pirazocillin and other semisynthetic penicillins. The penicillin-inactivating action of compounds was studied by means of adding their solutions dissolved in 1/4 M phosphate buffer at a final concentration of 5×10^{-6} M/ml to a penicillinase solution of 50 IU/ml concentration. The mixture was incubated at 37°C, and at intervals the amount of penicillanic acid formed was determined by iodometric titration. Spontaneous degradation was negligible. To the samples 1 ml of penicillin-G (100,000 IU/ml dissolved in phosphate buffer pH 6.5) was added and after further incubation at 37°C for 10 min, the amount of penicillanic acid formed was repeatedly determined iodometrically. In control experiments the amount of penicillanic acid formed in a penicillin-G solution of 100,000 IU/ml concentration on the effect of 50 IU/ml penicillinase was determined. In Fig. 15 the amount of penicillanic acid formed in the presence of the penicillins tested is indicated in per cents, where the amount of penicillanic acid formed in a solution containing penicillin-G only was taken as 100% peni-

cillinase activity. The amount of penicillanic acid formed from the various penicillins due to spontaneous decomposition was taken into correction.

Protection tests in mice. The 50% curative dose (ED_{50}) was determined for a variety of Gram-positive bacterial infections in 18–22 g white mice (CFLP, Budapest, Hungary). The infection was usually performed intraperitoneally but in the case of staphylococci producing penicillinase, intravenously. Care was taken to inject a sufficient number of bacterial cells to kill all mice belonging to the same group within 48 hr. In tests performed with penicillinase-producing staphylococci the treated mice were killed after 4–5 days. For infection the following microorganisms were used: β -haemolytic *Streptococcus* 33 ($LD_{100} = 5 \times 10^3$ cells/ml, own collection), *S. aureus* 1115 ($LD_{100} = 10^7$ cells/ml), penicillin-resistant *S. aureus* 53 ($LD_{100} = 10^8$ cells/ml resistant to penicillin-G, streptomycin, chloramphenicol, oxytetracycline, erythromycin and polymyxin), *S. aureus* Smith ($LD_{100} = 10^2$ cells/ml in suspension containing 5% mucin).

Antibiotics were administered in 0.5 ml volume, through a stomach tube or injected under the skin of the back, usually immediately after the infection. The number of mice belonging to each group was at least 10. Lethality was checked daily. Tests were stopped as a rule on the 7th day after infection. Then the number of surviving mice was recorded, the ED_{50} estimated by means of log-probit plot according to LITCHFIELD and WILCOXON [11]. Food and water were offered *ad libitum*.

Absorption studies. In part of the blood level studies we used white mice ranging in weight from 18 to 22 g. In oral absorption experiments mice were fasted for 18 hr prior to administration of the drug. Blood samples were obtained from the tail vein previously made hyperaemic. To estimate the blood antibiotic concentration, a filter paper-disk 5.0 mm in diameter previously sterilized by heat, was touched to the blood sample until saturation, kept at -4°C until all the samples had been prepared and then the disks were placed each on the surface of an agar plate prepared by the agar-diffusion test. Diameters of inhibition zones were determined after incubating the plates at 37°C for 18 hr. The antibiotic content was determined by the aid of a standard curve obtained as follows: in the heparinized blood of a non-treated mouse, a known amount of antibiotic was dissolved and the inhibition zones were plotted against the concentrations.

In experiments performed in dogs food was withdrawn for 18 hr before drug administration, but water was given *ad libitum*. In the case of oral administration, the amount of drug calculated for weight was administered in a capsule. At intervals, from venous blood samples collected under sterile conditions [4] the serum was separated and its biological activity was determined by the agar-diffusion test. In this case the dose-response-curve was constructed on the basis of dog serum containing a known amount of antibiotic.

Results

Studies in vitro. The *in vitro* antibacterial spectrum of Pirazocillin is demonstrated in Table II. The compound even at low concentrations exhibited an excellent activity against Gram-positive bacteria including penicillin-resistant staphylococci. It was ineffective against Gram-negative bacteria. In Dubos medium the compound exhibited a considerable inhibitory effect on the mycobacterium tested, while it did not exert any influence on the multiplication of this same microorganism in a medium free of Tween-80. It failed to inhibit the multiplication of the tested fungi. Its effectivity against Gram-

Key to Table II

I = penicillin-G-Na; II = streptomycin- SO_4 ; III = chloramphenicol; IV = oxytetracycline; V = chlortetracycline; VI = erythromycin; VII = polymyxin-D; VIII = oxacillin- $\text{Na} \cdot \text{H}_2\text{O}$

* the medium contained one drop of bovine serum per test tube

** Dubos medium containing Tween-80 [4]

*** Kirchner medium (without Tween-80) [5]

**** Sabouraud-bouillon [5]

Table II

Activity in vitro of Pirazocillin and other penicillins on various microorganisms

Medium: CM 225 (Oxoid); inoculum: $1-5 \times 10^5$ cells/ml

Organism	Minimum inhibitory concentration, $\mu\text{g/ml}$			
	Pirazocillin	Oxacillin	Dicloxacillin	Penicillin G
<i>Pseudomonas aeruginosa</i>	500	500	500	500
<i>Escherichia coli</i> O111 Resistant I-VII	500	500	500	30
<i>Escherichia coli</i> 4R Resistant I-VI	125	500	500	60
<i>Escherichia coli</i> 6R Resistant I-VI	500	500	500	500
<i>Escherichia coli</i> K12	32	125	500	30
<i>Shigella sonnei</i>	500	500	500	60
<i>Salmonella typhi-murium</i> 52234	500	500	500	—
<i>Proteus vulgaris</i> XL	500	500	500	500
<i>Proteus mirabilis</i> F342	500	—	500	500
<i>Proteus rettgeri</i>	125	—	125	500
<i>Proteus morganii</i> 558	500	—	500	500
<i>Brucella bronchiseptica</i>	500	—	500	—
<i>Streptococcus pyogenes</i> Lilly*	0.31	—	1.25	0.06
<i>Streptococcus pyogenes</i> Aronson*	0.31	—	1.25	0.06
<i>Streptococcus pyogenes</i> Robb*	0.08	—	0.08	0.16
<i>Streptococcus pyogenes</i> 766*	0.08	—	0.16	0.08
<i>Streptococcus pyogenes</i> 33*	0.08	—	0.08	0.08
<i>Streptococcus pyogenes</i> 496*	0.16	—	0.16	0.16
<i>Diplococcus pneumoniae</i> *	0.16	—	0.32	0.5
<i>Streptococcus faecalis</i>	10.0	—	16.0	3.1
<i>Bacillus subtilis</i> ATCC 6633	0.08	0.16	0.32	0.008
<i>Sarcina subflava</i>	0.015	0.04	0.16	0.008
<i>Staphylococcus aureus</i> Smith	0.08	0.08	0.16	0.08
<i>Staphylococcus aureus</i> Duncan	0.16	0.4	0.16	0.16
<i>Staphylococcus aureus</i> 53 Resistant I-VII	0.16	0.16	0.16	100
<i>Staphylococcus aureus</i> 80/81 Resistant I	0.16	0.4	0.16	100
<i>Staphylococcus aureus</i> 1115 Resistant I	0.16	0.16	0.16	100
<i>Staphylococcus aureus</i> 1101 Resistant I	0.16	0.4	0.16	100
<i>Staphylococcus aureus</i> 724 Resistant I	0.32	0.4	0.16	100
<i>Staphylococcus aureus</i> 824 Resistant VIII	1.25	10.0	1.25	100
<i>Mycobacterium tuberculosis</i> H ₃₇ Rv**	1.25	6.2	1.25	100
<i>Mycobacterium tuberculosis</i> Ravenel***	100	100	100	100
<i>Micobacterium tuberculosis</i> Ravenel**	0.8	6.2	0.8	100
<i>Candida albicans</i> ****	100	100	100	100
<i>Trichophyton quinckeanum</i> ****	100	100	100	100

positive microorganisms sensitive to penicillin-G was almost one order less than that of penicillin-G.

Table III presents Pirazocillin activity against penicillin resistant *S. aureus* strains isolated from clinical specimens, comparing it with the effect of penicillin-G and isoxazolyl penicillins. In the corresponding concentrations,

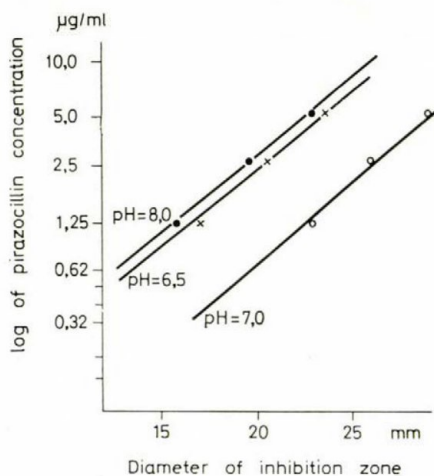


Fig. 3. Effect of pH of solution and medium upon Pirazocillin activity. *S. aureus* P-209 (penicillin sensitive) strain was used as indicator. In the 10 mm wells of the agar plate 0.1 ml volumes of Pirazocillin dilutions have been measured. Their concentration and the inhibition zones are shown

Pirazocillin inhibited the multiplication of more *S. aureus* strains than cloxacillin, whereas penicillin-G remained ineffective on the majority of these strains. Pirazocillin was bioassayed by the agar-diffusion method. A logarithmic correlation was established between the diameter of inhibition zones caused by the antibiotic concentration of the solution and that of the given concentration of the Pirazocillin solution. Effectivity was influenced by the pH of both solution and medium. As indicated in Fig. 3, when the same indicator strain was employed, Pirazocillin was most effective at neutral pH.

The relative potency of Pirazocillin and dicloxacillin was compared with strains of penicillin-sensitive *S. aureus* P209 (Fig. 4), penicillin resistant *S. aureus* 53 (Fig. 5) and with the spore suspension of the penicillin sensitive *B. subtilis* ATCC 6633 strain (Fig. 5), serving as indicator. The results obtained at pH 7.0 demonstrated no appreciable difference in the response of staphylococci to the two antibiotics, but when tested against *B. subtilis* ATCC 6633, Pirazocillin was nearly four times more potent than dicloxacillin.

The bactericidal effect of Pirazocillin was exerted solely on multiplying cells in the logarithmic phase. A concentration of 1.0 $\mu\text{g/ml}$ was sufficient

Table III

Susceptibility to different penicillins of *S. aureus* strains of clinical isolatesMedium: CM 225 (Oxoid); inoculum: $1-5 \times 10^5$ cells/ml

Concentration $\mu\text{g/ml}$	No. of strains inhibited					
	after 24 hr by			after 72 hr by		
	Pirazocillin	Cloxacillin	Penicillin-G	Pirazocillin	Cloxacillin	Penicillin-G
0.001	—	—	—	—	—	—
0.010	—	—	—	—	—	—
0.040	—	—	—	—	—	—
0.080	—	—	—	—	—	—
0.100	—	—	12	—	—	—
0.160	30	17	—	5	4	—
0.320	64	78	—	73	39	—
0.640	—	—	—	12	44	—
1.000	—	—	11	—	—	4
1.250	—	—	—	4	2	—
2.500	—	—	—	—	2	—
5.00	—	—	—	—	1	—
10.000	—	—	21	—	—	12
100.000	—	—	48	—	—	11
500.000	—	—	—	—	—	65
1000.000	—	—	—	—	—	1
Total	94	95	92	94	92	93

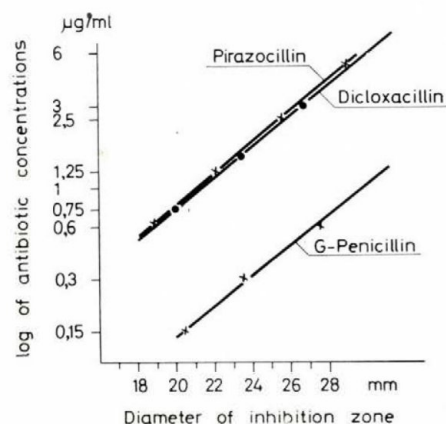


Fig. 4. Potency of Pirazocillin, dicloxacillin and penicillin-G by agar-diffusion test. Indicator organism: penicillin-sensitive *S. aureus* P-209

to kill all living bacteria of the culture within 24 hr, even in the case of the penicillin resistant *S. aureus* 53 strain, as shown in Fig. 5.

Semi-logarithmic plots of viable cell numbers present after exposure of bacteria to different concentrations of Pirazocillin for various periods are illustrated in Fig. 6. At low concentrations such as 0.1 $\mu\text{g/ml}$, the compound initially decreased the number of viable cells but the number of survivors was obviously sufficient to permit resumption of growth at some point between 5–26 hr. Growth was just visible at 24 hr in tubes containing 0.1 $\mu\text{g/ml}$ Pirazocillin, whereas in those which contained 1.0 $\mu\text{g/ml}$ or more, no viable cells could be detected. Dicloxacillin showed a similar effect. In the stationary phase neither of these antibiotics exerted a bactericidal effect and no measurable change was detectable in the number of viable cells (Fig. 7).

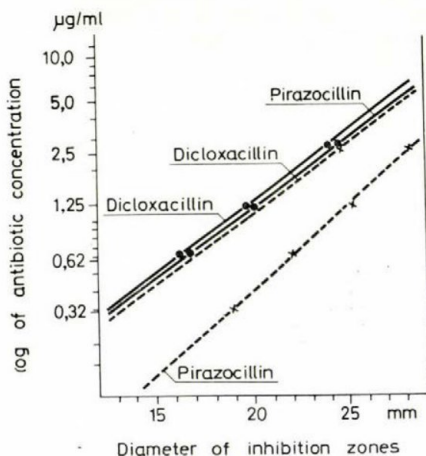


Fig. 5. Potency of Pirazocillin and dicloxacillin by the agar-diffusion test against *B. subtilis* ATCC 6633 (— — —) and penicillin resistant *S. aureus* 53 (—)

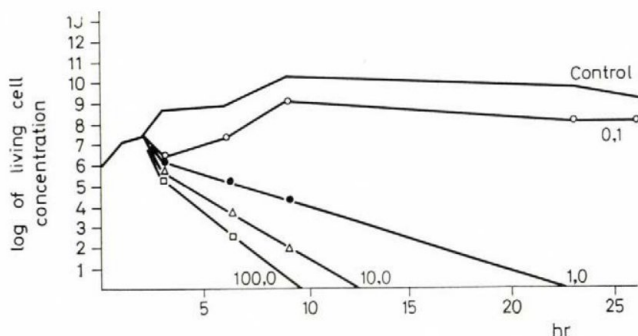


Fig. 6. Bactericidal effect of Pirazocillin on penicillin resistant *S. aureus* 53 in the logarithmic phase. The number of live organisms isolated from the Pirazocillin-containing medium at different times are indicated

In order to obtain data on the occurrence of penicillin resistant cells in *S. aureus* 53, into media containing geometrically increasing antibiotic concentrations 0.5 ml from the suspension of just viable cells were inoculated. It is demonstrated in Fig. 8 that in the case of Pirazocillin the development of resistant cells was somewhat delayed.

On the basis of the chemical similarity between oxacillin and Pirazocillin we have studied whether an oxacillin resistant *S. aureus* 824 strain showed cross-resistance against Pirazocillin. In the course of the study, two variants of this strain were tested, in view of the frequent reversibility of resistance. In the experiments strains transferred 25 times prior to employment and maintained on either 500 $\mu\text{g}/\text{ml}$ oxacillin containing agar medium (Bacto No. 110 with 1.5% agar content) (A), or on drug free medium (B) were tested. From a suspension (or its dilution) cultivated on liquid medium at 37°C, for 18 hr, 1.0 ml was spread over the agar surface and after further incubation at 37°C for 18 hr cells were transferred by replication [8] onto solid medium containing 100, 200, 300 and 400 $\mu\text{g}/\text{ml}$ of antibiotic. The number of colonies formed

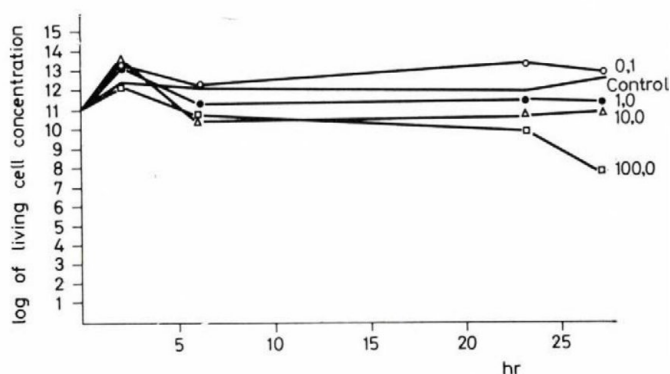


Fig. 7. Bactericidal effect of Pirazocillin on penicillin resistant *S. aureus* 53 strain in the stationary phase. The number of live organisms isolated from the Pirazocillin-containing medium at different times are indicated

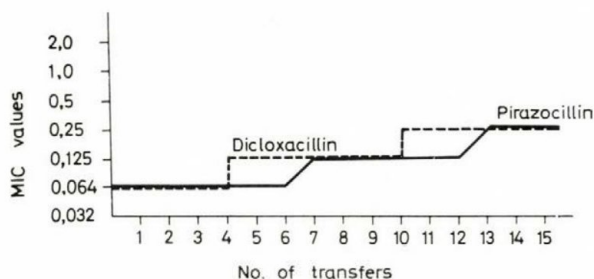


Fig. 8. Sensitivity of *S. aureus* 53 strain in the course of serial transfers into Pirazocillin-containing medium: period of transfer, 24 hr at 37°C

at 37°C in 24 and 72 hr in the presence of oxacillin (Fig. 9), Pirazocillin (Fig. 10) and penicillin-G (Fig. 11) were counted. Results were expressed in per cents of the control test (number of colonies formed on agar plate free of drug). It can be seen that the strain tested was actually resistant against oxacillin, whether or not it was maintained on oxacillin containing (A) medium or on one free of drug (B). Pirazocillin proved to be most effective among the antibiotics tested. A slight difference of Pirazocillin susceptibility between strains cultured in (A) and (B) was detectable after 24 hr but this difference ceased after 72 hr. Pirazocillin inhibited the multiplication of oxacillin-resistant strains

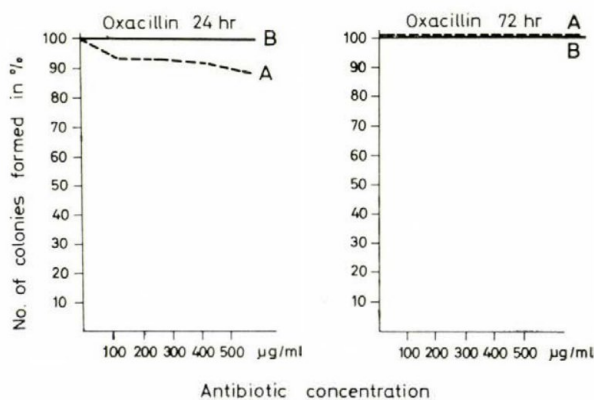


Fig. 9. Effect of oxacillin on the multiplication of oxacillin resistant *S. aureus* 824 after incubation at 37°C for 24 and 72 hr. *S. aureus* 824 maintained on oxacillin-containing (A) medium and on medium free of drug (B) was inoculated by replication into media containing different concentrations of oxacillin. The number of colonies formed after 24 and 72 hr incubation was counted and indicated in per cent of those formed in medium free of drug

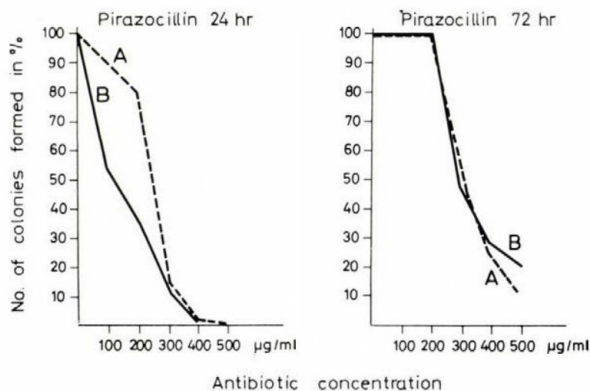


Fig. 10. Effect of Pirazocillin on the multiplication of oxacillin resistant *S. aureus* 824 after 24 and 72 hr incubation at 37°C. The strain was maintained on oxacillin-containing (A) medium and on medium free of drug (B). Both were inoculated by replication into media containing different concentrations of Pirazocillin. The number of colonies formed at 37°C after 24 and 72 hr was counted and indicated in per cent of those formed in medium free of drug

only at high concentrations, at least 200 $\mu\text{g/ml}$, as a practical proof of the cross-resistance existing between the two compounds. The number of viable cells was not markedly decreased even by large doses of oxacillin and penicillin-G.

Since the biological activity of Pirazocillin in aqueous solution did not decrease at neutral pH at 37°C within 6 hr, therefore its stability at low pH

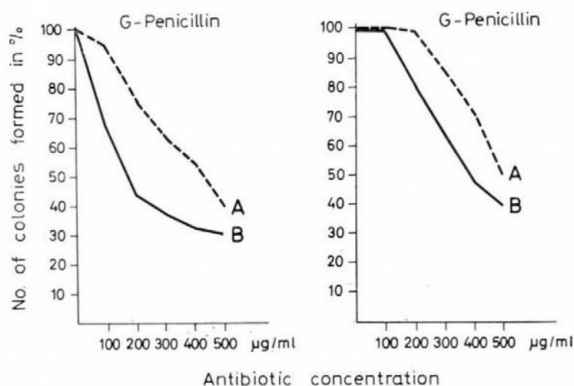


Fig. 11. Effect of penicillin-G on the multiplication of oxacillin resistant *S. aureus* 824 strain after incubation at 37°C for 24 and 72 hr. The strain was maintained on oxacillin containing (A) medium and on medium free of drug (B). They were inoculated by replication into media having different concentrations of penicillin-G. The number of colonies formed after 24 and 72 hr incubation at 37°C was counted and indicated in per cent of those formed in medium free of drug

has also been studied. Fig. 12 shows the residual bioactivity of the compound and that of isoxazolyl penicillin derivatives after exposure to pH 2.0 buffer for various periods.

In artificial gastric juice (pH 1.3) at 37°C the biological activity of the compounds varied even more markedly. Related data are given in Fig. 13. These results indicate that the binding of Pirazocillin to macromolecules (mucin) also decreased its effect.

Binding of Pirazocillin to serum proteins is demonstrated in Fig. 14. Although the extent to which the tested penicillins are bound to serum proteins was considerable, the residual part of Pirazocillin was nearly 3 times as much that of a dicloxacillin recovered under the same conditions.

Pirazocillin was resistant against the effect of β -lactamase of *B. cereus* origin (Fig. 15). In the course of 2 hr, 97% of oxacillin, 93% methicillin, whereas only 10% of dicloxacillin and 9% of Pirazocillin was decomposed by the enzyme. In control experiments, spontaneous decomposition of the compounds was negligible. Thus, Pirazocillin proved to be extremely stable against the effect of β -lactamase and in this respect it was superior to numerous "penicillin resistant" compounds and it was nearly identical to dicloxacillin in this respect.

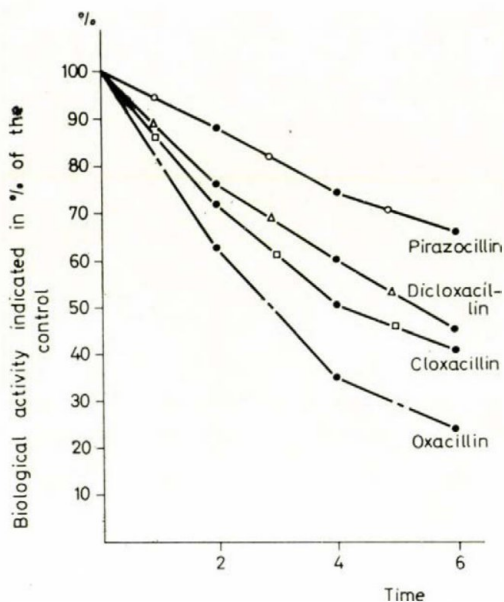


Fig. 12. Decomposition of various semisynthetic penicillins in pH 2.0 glycine buffer at 250 $\mu\text{g/ml}$ concentration, 37°C. Bioassay was done after neutralization by agar-diffusion test using spore suspension of *B. subtilis* ATCC 6633 as indicator

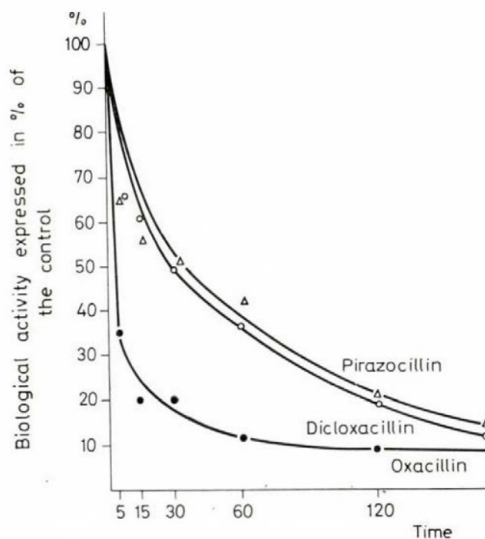


Fig. 13. Decomposition of various semisynthetic penicillins in artificial gastric juice pH 1.3 at 700 $\mu\text{g/ml}$ concentration, 37°C. Biological activity of the residue was determined after neutralization by agar-diffusion test, using *B. subtilis* ATCC 6633 as indicator

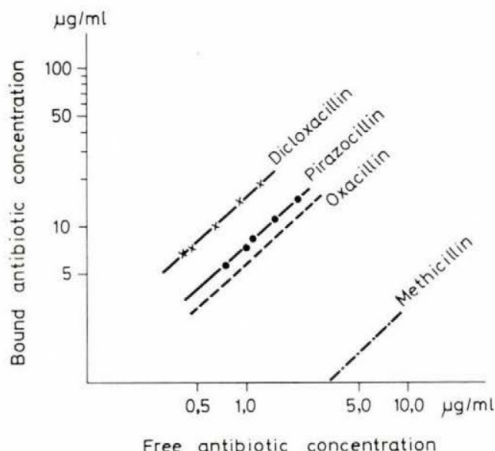


Fig. 14. Binding of semisynthetic penicillins to serum proteins, according to SCHOLTAN and SCHMID [10]. Log concentrations of free and bound antibiotic ranging between 5–20 $\mu\text{g/ml}$ are indicated

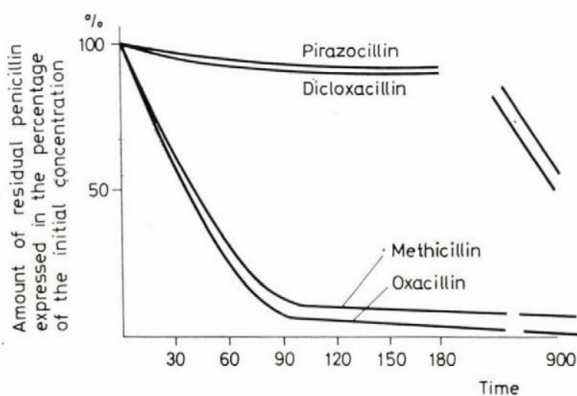


Fig. 15. Decomposition of semisynthetic penicillins by penicillinase. The amount of penicillanic acid formed from the various penicillin solutions at 5×10^{-6} M/ml concentration under the effect of 50 IU/ml penicillinase was determined iodometrically. The original penicillin concentrations titrated at different times are shown

In earlier studies [12] we have demonstrated that Pirazocillin not only inhibited the action of penicillinase but inactivated it as well which could be explained by the conformational change of the enzyme [13]. In contrast to the other semisynthetic penicillins, tested simultaneously, Pirazocillin exerted a strong and prolonged inhibitory effect against penicillinase, similarly dicloxacillin (Fig. 16).

Studies in vivo. The strains of bacteria usually employed for experimental infections showed a considerable susceptibility to Pirazocillin *in vitro*.

The virulence of *Streptococcus pyogenes* strain 33 (own collection) on mice was moderate (Table IV). The infected mice responded well to antibiotic treatment (Table V). There was no measurable difference in effect between orally administered Pirazocillin or dicloxacillin immediately after infection and for the following 2 days. Under similar experimental conditions the effect of Pirazocillin and cloxacillin was compared by adding the drugs orally to

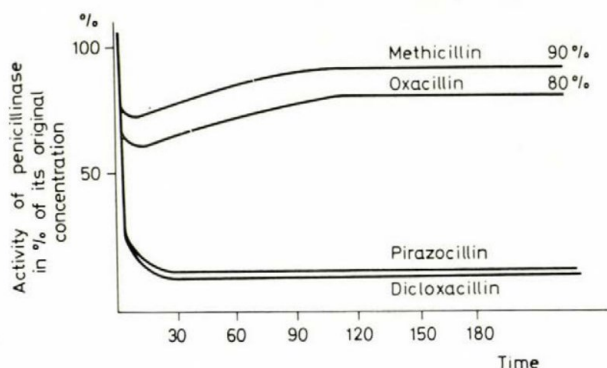


Fig. 16. Activity of penicillinase from *B. cereus* 569 H under the effect of different penicillins. To penicillinase solution of 50 IU/ml concentration penicillin solutions were added to allow a final antibiotic concentration of 5×10^{-6} M/ml. The mixture was incubated at 37°C, and at intervals the amount of penicillanic acid formed was determined iodometrically. To samples taken at the same intervals 1 ml of penicillin-G (100 000 IU/ml) was added and after further incubation at 37°C for 10 min the formed amount of penicillanic acid was determined iodometrically. In control experiments the amount of penicillanic acid formed in a penicillin-G solution of 1 000 000 IU/ml concentration under the effect of 50 IU/ml penicillinase was determined. The amount of penicillanic acid formed in the presence of penicillins is indicated in per cent, where the amount of penicillanic acid formed in solution containing penicillin-G only was regarded as characteristic of 100% penicillinase activity. The amount of penicillanic acid formed from the various penicillins by spontaneous decomposition was taken into correction

Table IV

Pathogenicity in mice of S. pyogenes 33

Intravenous infection with 6 hr culture containing 10^7 cells/ml: medium, CM 225 (Oxoid) + one drop of bovine serum

Infecting cell number	No. of mice	Average body weight, g	No. of survivors		
			1	2	3
			days after infection		
5×10^5	5	17.5	—	—	—
5×10^4	5	17.5	1	—	—
5×10^3	5	18.0	1	—	—
5×10^2	5	18.0	4	1	1
5×10	5	18.0	5	2	2

mice on one single occasion. When the drugs were administered 1 hr prior to infection, cloxacillin, while 1 hr after infection, Pirazocillin was found to be more effective. This was manifest in the differences in ED_{50} values.

The effect of Pirazocillin and dicloxacillin was compared in mice infected with *S. aureus* Smith, a non-penicillinase producer strain (Table VI). Regardless of the route of treatment, Pirazocillin was superior to dicloxacillin, but the difference in ED_{50} values was not significant statistically.

The pathogenicity in mice of the penicillinase resistant *S. aureus* strains tested was very low. Table VII summarizes the results obtained with *S. aureus* 1115 after intravenous infection. The effect of Pirazocillin and isoxazoly penicillins was compared in mice infected with these strains. It is apparent from

Table V

Effect of Pirazocillin and isoxazoly penicillins in mice

intraperitoneal infection with *S. pyogenes* 33 with $10 \times LD_{100} = 5 \times 10^4$ cells/mouse; observations period, 7 days

Drug	Treatment schedule	No. of applications	No. of mice	ED_{50} mg/kg
Pirazocillin	daily after infection	3	10	8.5 (3.5–16)*
Dicloxacillin	daily after infection	3	10	8.5 (3.5–16)
Pirazocillin	1 hr before infection	1	10	15
Cloxacillin	1 hr before infection	1	10	10
Pirazocillin	1 hr after infection	1	10	80
Cloxacillin	1 hr after infection	1	10	120

* 84.13% probit limits

Table VI

Effect of Pirazocillin and dicloxacillin in mice infected with penicillin sensitive S. aureus Smith strain

Intraperitoneal infection with $10 \times LD_{100} = 10^3$ cells/mouse in aqueous suspension containing 5% mucin; observation period, 7 days

Drug	Route	No. of applications	No. of mice	ED_{50} mg/kg
Pirazocillin	intravenous	1	10	16 (3–80)*
Dicloxacillin	intravenous	1	10	20 (3–120)
Pirazocillin	subcutaneous	1	10	5 (0.5–21)
Dicloxacillin	subcutaneous	1	10	32 (4–150)
Pirazocillin	oral	1	10	26 (14–42)
Dicloxacillin	oral	1	10	32 (16–66)

* 84.13% probit limits

Table VIII that ED_{50} values were generally higher in mice infected with penicillinase producer *S. aureus* 1115 but Pirazocillin was still the most effective among the compounds. Its potency was unchanged when simultaneously with the infection 50 IU of penicillinase had been administered. The differences between Pirazocillin and dicloxacillin are illustrated in characteristic experiments (Tables IX and X).

Thus protection experiments revealed that Pirazocillin not only protected mice against *S. pyogenes* or *S. aureus* but also against otherwise lethal infection with penicillin resistant *S. aureus* strains. The ED_{50} values were

Table VII

Pathogenicity in mice of penicillin resistant S. aureus 1115

Intraperitoneal infection with 0.5 ml culture

Dose	Mice		No. of survivors											
	No.	Average weight, g	0	1	2	3	4	5	6	7	8	9	10	11
			days after infection											
5×10^8	10	18.1	10	5	1	1	0	0	0	—	0	0	0	0
5×10^7	10	20.3	10	9	7	6	3	3	3	—	3	3	3	3
5×10^6	10	20.5	10	9	9	8	7	6	4	—	4	4	4	4
5×10^5	10	18.9	10	10	10	10	9	9	9	—	9	9	8	7
5×10^4	10	17.6	10	10	10	9	9	9	9	—	8	8	8	8

Table VIII

Effect of Pirazocillin and isoxazolyl penicillins

Intravenous infection with penicillin resistant *S. aureus* 1115; $1 \times LD_{100} = 10^7$ cells/ml; observation period, 7 days

Compound	Route of treatment	No.	No. of mice	ED_{50} mg/kg
Pirazocillin	intraperitoneal	1	10	35 (20–75)*
Oxacillin	intraperitoneal	1	10	60 (15–250)
Pirazocillin	intraperitoneal	3	10	13 (4–40)
Cloxacillin	intraperitoneal	3	10	25 (6.5–100)
Pirazocillin	oral	3	10	45 (13–140)
Cloxacillin	oral	3	10	65 (22–200)
Pirazocillin	intraperitoneal	1	10	30 (15–70)
Dicloxacillin	intraperitoneal	1	10	50 (14–160)
Pirazocillin	oral	1	10	38 (22–80)
Dicloxacillin	oral	1	10	65 (27–150)

* 84.13% probit limits

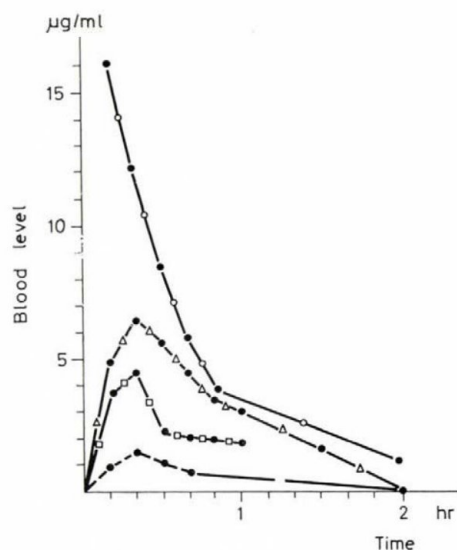


Fig. 17. Mean blood levels in mice after Pirazocillin administration; 5 mg/kg subcutaneously —; 50 mg/kg intravenously —○—; 50 mg/kg orally —●—; 100 mg/kg orally —□—

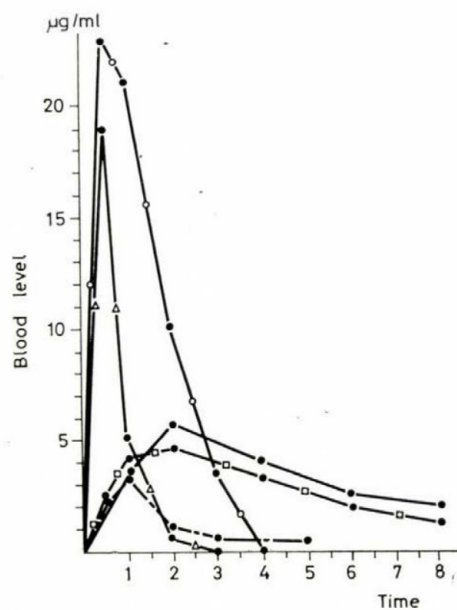


Fig. 18. Mean blood levels in dogs after Pirazocillin administration: 25 mg/kg subcutaneously —△—; 50 mg/kg subcutaneously —○—; 100 mg/kg orally —□—; 300 mg/kg orally —

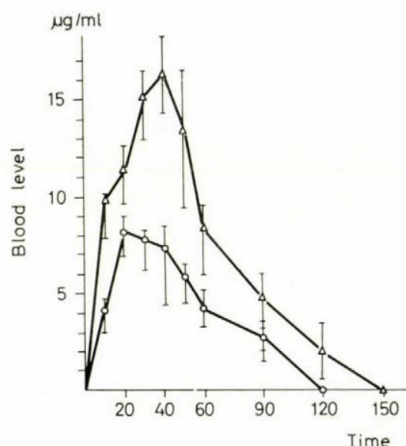


Fig. 19. Blood Pirazocillin and dicloxacillin levels in mice after oral administration of 100 mg/kg. In each case 14 mice were tested. Pirazocillin —○—; Dicloxacillin —●—

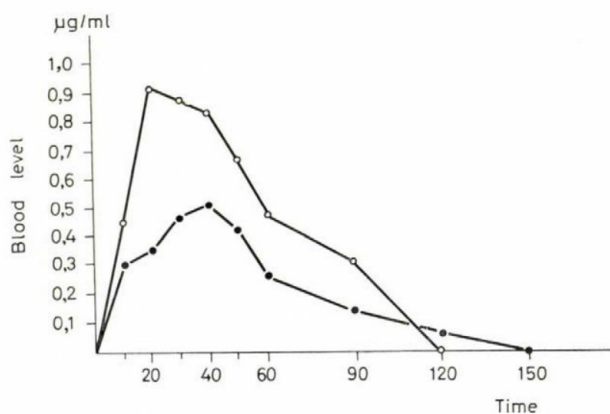


Fig. 20. Free antibiotic concentration calculated from blood levels in mice after oral administration of 100 mg/kg of Pirazocillin and dicloxacillin. In each case 14 mice were tested. Pirazocillin —○—; Dicloxacillin —●—

Table IX

Effect of Pirazocillin and isoxazolylic penicillins

Intravenous infection with $1 \times LD_{100} = 10^7$ cells/ml of *S. aureus* 1115 + 50 IU of penicillinase; observation period, 7 days

Compound	Route of treatment	No.	No. of mice	ED ₅₀ mg/kg
Pirazocillin	oral	1	10	35 (16-70)*
Dicloxacillin	oral	1	10	65 (27-150)

* 84.13% probit limits

usually lowest with Pirazocillin but no measurable divergences from those obtained with dicloxacillin were detectable on oral or venous administration. On subcutaneous injection Pirazocillin proved superior to dicloxacillin.

Several experiments were carried out to investigate the absorption of the compounds. Results obtained in mice are collected in Fig. 17. After intravenous administration of 50 mg/kg Pirazocillin, the drug concentration in serum gradually decreased and 2 hr later effective blood levels were measured. An equal amount administered orally resulted in considerably lower blood levels and effectivity was ensured only for 1 hr. Twofold doses caused twofold blood levels.

In dogs, 50 mg/kg Pirazocillin administered orally induced an inhibitory blood level for a period of 2 hr. For its maintainance or for higher blood levels, proportionally higher doses were required. Active blood levels persisted longer in dogs than in mice. Intramuscular administration of Pirazocillin was followed by higher blood levels in dogs, too (Fig. 18) but for a shorter period than after oral dosage.

On oral administration, Pirazocillin caused lower blood levels than did dicloxacillin (Fig. 19). Considering, however, the different extent to which

Table X

Effect of Pirazocillin and dicloxacillin

Intravenous infection with $1 \times LD_{100} = 5 \times 10^8$ cells/mouse of penicillin resistant *S. aureus* 53; observation period, 7 days

Compound	Route of treatment	No.	No. of mice	ED ₅₀ mg/kg
Pirazocillin	oral	1	10	38 (22–80)*
Dicloxacillin	oral	1	10	65 (31–103)

* 84.13% probit limits

Table XI

Free antibiotic concentration in blood after oral administration of 100 mg/kg Pirazocillin and dicloxacillin

Compound	Time of blood sampling, min								
	10	20	30	40	50	60	90	120	150
Pirazocillin									
total determined	4	8.2	7.8	7.4	5.9	4.2	2.8	0	0
free calculated	0.44	0.91	0.87	0.82	0.66	0.47	0.31	0	0
Dicloxacillin									
total determined	9.8	11.4	15.2	16.4	13.4	8.4	4.7	2.0	0
free calculated	0.3	0.35	0.47	0.51	0.42	0.26	0.14	0.06	0

Calculation of free antibiotic concentration was based on the amount of Pirazocillin (11.1%) and dicloxacillin (3.1%) measured by SCHOLTAN's and SCHMID's method [10] in the presence of serum

the two antibiotics are bound to serum proteins, the free drug concentration calculated on this basis (Table XI) is higher in the case of Pirazocillin. This might be an explanation for the greater potency of Pirazocillin *in vivo* (Fig. 20), since this depends on the concentration of free drug [14].

Discussion

Substitution of the "side-chain" of penicillin-G by chemical means was made possible by the isolation and chemical identification of 6-APA [15]. By linking to 6-APA different organic acids, new penicillin analogues possessing different chemotherapeutic and chemical properties could be produced [16]. Some of these compounds are widely used as they have solved some difficulties arising in the course of treatment with penicillin-G and V. These semisynthetic penicillins can be administered orally, they are rapidly absorbed, have a prolonged effect, are not toxic and prove resistant to penicillinase [17].

According to the present results, Pirazocillin meets all these requirements. In its chemical structure corresponds to the principles emphasized by DOYLE and NAYLER [16], who concluded that the resistance of the molecule to acid and penicillinase is considerably enhanced if the alpha carbon atom in carbonic acid bound to 6-APA by an amide is substituted by an aliphatic radical, in our case a dichlorophenyl group. In this respect, Pirazocillin and dicloxacillin may be considered analogous substances [18], and in fact they are closely similar in their chemotherapeutic potency. The greatest difference between the two agents was observed in the rate of binding serum proteins and in the rate of absorption following oral administration. This difference may be attributed to the different nature of the isoxazole and pirazole rings.

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GENETIC BEHAVIOUR OF THE *colE1-ML* FACTOR

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Summary. The genetic behaviour of the *colE1-ML* determinant is the same as that known for *colE1-K30*. It has no sex factor activity but a fertility factor such as F, renders its transfer efficient. Its genetic transfer does not interfere with the F promoted chromosomal transfer, nor does it appear to interfere with the chromosomal recombination process in the recipient bacteria. Preliminary results suggest, however, that the chromosomal transfer or the recombination process could interfere with the plasmid replication.

Our knowledge of the *colE1* determinant is based exclusively on the study of the *colE1-K30* plasmid [1-5]. Although this self-dependent circular entity has no fertility factor activity, any fertility factor such as F renders its transfer from bacteria to bacteria highly efficient. This transfer does not interfere with the F promoted chromosomal transfer, nor does it appear to interfere with the chromosomal recombination process in the recipient bacteria [1-5].

The *colE1* determinant of *Escherichia coli* strain ML is a strongly established genetic element that does not promote its own transfer to other strains. Moreover, there is an incompatibility barrier between the strain *E. coli* ML and that of the *E. coli* K12 line, which renders it difficult to construct genetic systems with *colE1-ML* determinant in the *E. coli* K12 line of bacteria.

In one particular case, however, we could transfer the *colE1* determinant directly from the *E. coli* ML strain into the *E. coli* K12 line. We used this strain to obtain data on the genetic behaviour of the *colE1-ML* determinant.

Materials and methods

Bacterial strains. The strains, their relevant characters, and their origin are listed in Table I.

Media. (a) Tris minimal medium: NH_4Cl , 1 g; Tris, 12 g; KCl, 0.035 g; $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.2 g; NaCl, 0.058 g; $\text{Na}_2\text{SO}_4 \cdot 10 \text{H}_2\text{O}$, 0.3 g; KH_2PO_4 , 0.14 g; glucose, 2.0 g; distilled water, to 1000 ml; pH 7.5.

(b) Tryptone yeast extract enriched medium: NH_4Cl , 1 g; K_2HPO_4 , 7.0 g; NaH_2PO_4 , 3.0 g; Bacto Tryptone (Difco), 10 g; Yeast Extract (Difco), 1.0 g; NaCl, 8.0 g; distilled water 1000 ml; pH 7.2.

Genetic crosses. Donor and recipient bacteria, grown in tryptone-yeast extract enriched medium, in the logarithmic state of growth were centrifuged, washed and resuspended in fresh tryptone-yeast extract enriched medium. They were mixed at a ratio of 1 Hfr to 20 F-

Table I
Relevant characters of strains used

Code	Strain	Sex	Colicin determinant or resistance	Auxotrophy	Strepto-mycin	Origin
LA 3100	<i>E. coli</i> ML	—	<i>EI</i>	<i>ile, val</i>	s	E. Wollman
LA 5000	<i>E. coli</i> AB1528	F(ile)	—	<i>his, pro, arg, BI</i>		A. E. Adelberg
LA 2600	<i>E. coli</i> Hfr-C	Hfr	—	<i>met, BI</i>	s	G. S. Stent
LA 2610	<i>E. coli</i> Hfr-C	Hfr	—	<i>met, ileA, BI</i>	s	This laboratory
LA 2611	<i>E. coli</i> Hfr-C	Hfr	<i>EI-ML^{res}</i>	<i>met, ileA, BI</i>	s	This laboratory
LA 2612	<i>E. coli</i> Hfr-C	Hfr	<i>colEI-ML</i>	<i>met, ileA, BI</i>	s	This laboratory
LA 1800	<i>E. coli</i> PA-309	F ⁻		<i>thre, leu, arg, his, try, BI</i>	r	G. S. Stent
LA 1810	<i>E. coli</i> PA-309	F ⁻	<i>EI-ML^{res}</i>	<i>thre, leu, arg, his, try, BI</i>	r	This laboratory
LA 1811	<i>E. coli</i> PA-309	F ⁻	<i>colEI-ML</i>	<i>thre, leu, arg, try, met, BI</i>	r	This laboratory

gently shaken in a 37°C water bath and after a 90 min incubation period seeded (without using soft agar) on selective media in order to reisolate the parents and the recombinants.

Demonstration of colicinogeny. Colicin production was demonstrated either on the selection plates or after transfer of the colonies to tryptone-yeast extract enriched medium. The plates were seeded with a liquid culture of the indicator bacterium and dried before closing. The inhibition around the colonies was checked after 16 hr.

Results

Transfer of the *colEI-ML* determinant into *E. coli* K12 line. After unsuccessful trials with different techniques and various *E. coli* K12 derivatives, we have finally transferred the *colEI-ML* determinant of the ML strain into the *E. coli* Hfr-C derivative of *E. coli* K12 strain as follows. The *E. coli* Hfr-C *met⁻, ile⁻* (LA 2610) strain was phenocopied to F⁻ by vigorous shaking and mixed with *E. coli* AB 1528, F(ile) (LA 5000). Then *ile⁺ met⁻* recombinants were selected. From the *E. coli* Hfr-C F(ile), *met⁻* bacteria, *EI-ML* resistant mutants were isolated and then incubated together with the *E. coli* ML strain in a liquid tryptone-yeast extract enriched medium. From this mixed culture we isolated a colicinogen *E. coli* Hfr-C, which had lost its F(ile) plasmid. Since that time the strain has been colicinogenic and we designate it as *E. coli* Hfr-C, (*colEI-ML*), *met⁻, ile⁻* (LA 2612).

Transfer of the *colEI-ML* determinant in *Hfr-col⁺ × F⁻ col⁻* crosses. The *colEI-ML* determinant of strain LA 2612 is easily transferred to any F⁻ and in these crosses it behaves as an autonomous genetic element without sex factor activity.

When the recipient parent bacteria are reisolated after a 90 min mating period from an Hfr *col⁺ × F⁻ col⁻* cross, the number of colicinogenic colonies is roughly equal to the number of minority parent Hfr bacteria present. This fact clearly demonstrates that the *colEI-ML* determinant's transfer from the

Hfr is very effective, and that this determinant is unable to spread among the F^- bacteria. Furthermore, stably *colEI-ML* F^- bacteria cannot transfer the *col* determinant to other F^- strains.

Distribution of the *col* determinant among the different classes of chromosomal recombinants does not show any linkage; the frequency of colicinogeny is 40–60% independently of the marker selected.

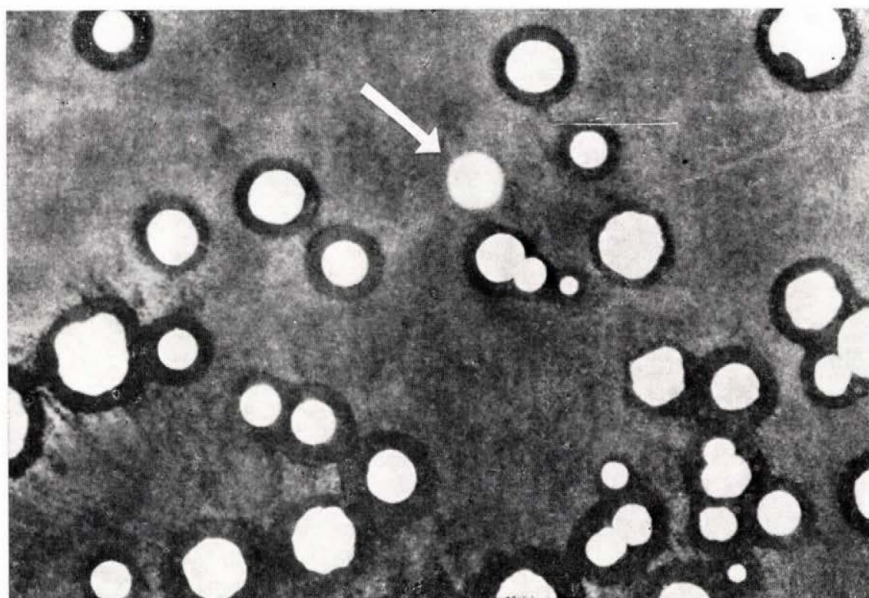


Fig. 1. *col*⁻ colony among the *arg*⁺ recombinants of the *Hfr col*⁻ × *F*⁻ *col*⁺

Loss of colEI-ML determinant from the progeny of Hfr col⁻ × *F*⁻ *col*⁺

Presence of the *colEI-ML* determinant in a recipient bacterium does not interfere with the conjugation and recombination process. Normal frequency values were obtained with these recipients independently of the selected chromosomal recombinants.

Because plasmid type inheritance is not associated with the chromosome (*i.e.* it is strictly cytoplasmic) and because colicin production depends on plasmid inheritance, one would expect colicinogenic recipient bacteria to remain colicinogenic even after mating with a colicin-negative donor. In contrast, colicin-negative recombinants were found among the chromosomal recombinants of *E. coli* *Hfr-C col*⁻ (LA 2600) × *E. coli* *PA309 col*⁺ (LA 1811) cross (Fig. 1).

The loss of the *colEI-ML* determinant is not restricted to any class of the chromosomal recombinants, but may vary from 0.1 to 15%, depending

on the selection. Preliminary data indicate that the higher losses occur when selection is forced for more than a single chromosomal marker.

Discussion

The *colEI-ML* determinant's behaviour in our Hfr *col*⁺ × F⁻ *col*⁻ crosses was the same as that already known for the *colEI-K30* plasmid.

It is worthwhile to speculate on the unexpected appearance of colicin minus recombinants from the Hfr *col*⁻ × F⁻ *col*⁺ crosses. Although the presence of *colEI-ML* plasmid influences neither the F promoted chromosomal transfer nor the chromosomal recombination process, chromosomal transfer-recombination may differentially inhibit *colEI-ML* plasmid replication in *col*⁺ recipient bacteria, sometimes resulting in losses of colicinogeny. Therefore, results of the Hfr *col*⁺ × F⁻ *col*⁻ cross should be re-evaluated.

It is surprising that although each *colEI*⁺ donor is able to transfer its *col* determinant as judged from the reisolated recipient parent, only 40–60% of the chromosomal recombinants should be colicinogenic.

It has been established that the *colEI-K30* determinant is transferred into the recipients a few minutes, well before the transfer of chromosomal markers. We have found similar early transfers at the *colEI-ML* plasmid (E. K., unpublished observation), therefore, chromosomal transfer does not seem to interfere with *colEI* plasmid transfer.

Should, however, the chromosomal recombination process interfere with the multiplication of *colEI* plasmid, both the absence of the *colEI* determinant from some chromosomal recombinants of an Hfr *col*⁺ × F⁻ *col*⁻ cross, and the loss of *colEI-ML* plasmid from recombinants of Hfr *col*⁻ × F⁻ *col*⁺ cross, could be understood.

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EFFECT OF RIFAMYCIN AND ITS DERIVATIVES ON THE TRANSFER OF R FACTOR IN ESCHERICHIA COLI

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(Received March 4, 1974)

Summary. Rifampicin at concentrations of 0.19 to 3.0 $\mu\text{g/ml}$ inhibited the transfer of R factor from *E. coli* K₁₂ J5 RC 709 F⁻ *prol*⁻ *meth*⁻ *Sm*^r *T*^r *Su*^r to *E. coli* K₁₂ *Sm*^s *T*^s *Su*^s. Transfer frequency decreased parallel with the increase of rifampicin concentration, but more than 3 $\mu\text{g/ml}$ of rifampicin (6–12 $\mu\text{g/ml}$) also affected the growth of both donor and recipient cells. Rifamycin and rifo exerted similar inhibitory effects on the transfer of R factor. Curing activity was not exhibited by any of the three antibiotics tested.

Drug resistance mediated by R factor has been known to occur among *Enterobacteriaceae* since 1959 [1, 2]. The R factors are extrachromosomal genetic elements that replicate independently of the host chromosome and carry determinants for resistance to several antibiotics. The world-wide increase in the frequency of *Enterobacteriaceae* with multiple drug resistance represents a problem of theoretical and practical significance. Various substances have been shown to eliminate the R factor from the host cells. The curing activity of rifampicin has been described by KRŮMÉRY and JANOUŠKOVÁ [3].

The present paper deals with experiments in which the effect of rifamycin and its two derivatives has been studied on the transfer of the R factor between cells of *Escherichia coli*. The agents were tested also for curing activity.

Materials and methods

Bacterial strain. *E. coli* K₁₂ J5 RC 709 F⁻ *prol*⁻ *meth*⁻ streptomycin (*Sm*)^r, tetracycline (*T*)^r and sulphadimidine (*Su*)^r, received through the courtesy of Dr. N. DATTA, London, was used as donor strain and an *E. coli* K₁₂ *Sm*^s *T*^s *Su*^s served as recipient strain.

Culture media. YP complete medium contained yeast extract and peptone; M-9 medium was described previously [4]; MTY medium consisted of M-9, trypton and yeast extract. The M-9 medium supplemented with 25 $\mu\text{g/ml}$ streptomycin, 50 $\mu\text{g/ml}$ tetracycline and 400 $\mu\text{g/ml}$ sulphadimidine was used as selective medium.

Rifamycin and its derivatives. Rifamycin SV, rifampicin and rifo were the products of Lepetit Co., Milano. The antibiotics were dissolved following the method of Dr. I. LÁSZLÓ (Dept. of Ophthalmology, Szeged) in m/15 Na₂HPO₄ to which ascorbinic acid was added (2 mg/ml). The solutions were stored at 4°C in the dark and were used up to 25–30 days.

Transfer of R factor. One ml of donor and recipient cells taken from MTY medium after culturing for 16–18 hr was added to 10 ml MTY broth. The cultures were incubated on a shaking tray at 37°C for 6 hr then diluted in MTY to contain approximately 3×10^8 cells/ml. The donor (2 ml) was mixed with the recipient (2 ml) in a culture tube and incubated further without shaking at 37°C for 18 hr. As controls, donor and recipient cells at the same dilution were cultured separately. At the end of the conjugation period, 0.1 ml of dilutions of the mixed

culture were spread with glass rods on selective media. After 48 hr incubation the colonies were counted.

Frequency of transfer was expressed as a ratio of the number of R factor carrying recombinant cells to the number of recipient cells in 1 ml.

Effect of rifamycin and its derivatives on conjugation. Rifamycin and its derivatives were added at various concentrations to the mixtures of donor and recipient cells at the time of mixing. The effect of rifamycin and its derivatives was also tested on the growth of the donor and the recipient cells. After incubation at 37°C for 18 hr, 0.1 ml amounts of the mixed cultures were plated on selective media and the dilutions of donor and recipient cultures were tested on YP agar plates.

Estimation of cells which have lost the R factor. Donor cells were cultured with shaking at 37°C for 6 hr then diluted in MTY to about 3×10^8 cells/ml. Rifampicin (3 µg/ml), rifamycin (2 µg/ml) or rifo (2 µg/ml) were added to tubes containing 4 ml of donor cell dilutions. After 18 hr incubation at 37°C the cultures were diluted to 10^{-7} and 0.1 ml aliquots of these dilutions were inoculated on YP agar plates without antibiotics. Eighteen hours later the colonies were counted, then replica-plated on YP agar plates containing 25 µg/ml Sm, 50 µg/ml T and 400 µg/ml Su. On the next day the colonies were counted and their number was compared to that of colonies grown on the "master plates" without antibiotics.

Results

Transfer of R factor was inhibited by rifampicin, rifamycin and rifo (Tables I and II). Transfer frequency in the presence of drugs was decreased with 1 to 3 exponents as compared to that of the controls. Rifampicin at 3 µg/ml concentration caused a marked inhibition without any effect on the growth of either the donor or the recipient cells. At higher concentrations (6–12 µg/ml) a further decrease in the number of recombinants could be demonstrated, but the growth of bacterial cells was also inhibited. Rifamycin and rifo at 2 µg/ml concentration were effective in inhibiting, while they influenced slightly the viable count of bacterial cells.

The number of recombinants was estimated at different intervals after adding rifampicin at 3 µg/ml concentration. There were less recombinants (Fig. 1) in the presence than in the absence of rifampicin throughout the observation period. The difference was most pronounced after 18 hr of incubation.

Table I
Inhibition of R factor transfer by rifampicin

Rifampicin µg/ml	No. recipients/ml	No. of recombinants/ml	Frequency of transfer
0.000	2.0×10^9	8.0×10^4	4.0×10^{-5}
0.185	2.0×10^9	2.0×10^4	1.0×10^{-5}
0.375	1.8×10^9	3.5×10^3	2.0×10^{-6}
0.750	1.5×10^9	8.0×10^2	5.0×10^{-7}
1.500	1.0×10^9	1.0×10^2	1.0×10^{-7}
3.000	1.0×10^9	4.0×10^1	4.0×10^{-8}
6.000	8.0×10^8	2.0×10^1	2.5×10^{-8}
12.000	5.0×10^8	1.0×10^1	2.0×10^{-8}

Table II
Inhibition of R factor transfer by rifamycin and rifo

Antibiotic	Concentration, $\mu\text{g/ml}$	No. of recipients/ml	No. of recombinants/ml	Frequency of transfer
Rifamycin	0.00	2×10^9	8.0×10^4	4.0×10^{-5}
	0.25	2×10^9	8.0×10^3	4.0×10^{-6}
	0.50	2×10^9	2.0×10^3	1.0×10^{-6}
	1.00	1×10^9	6.0×10^2	6.0×10^{-7}
	2.00	1×10^9	8.0×10^1	8.0×10^{-8}
	4.00	8×10^8	1.0×10^1	1.2×10^{-8}
Rifo	0.00	2×10^9	8.0×10^4	4.0×10^{-5}
	0.25	2×10^9	1.2×10^3	6.0×10^{-7}
	0.50	1×10^9	2.0×10^2	2.0×10^{-7}
	1.00	5×10^8	4.0×10^1	8.0×10^{-8}
	2.00	2×10^8	1.0×10^1	5.0×10^{-8}
	4.00	1×10^8	no transfer	..

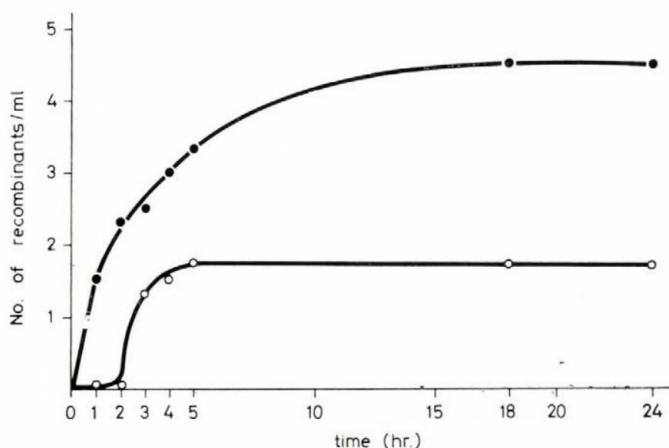


Fig. 1. Inhibition of recombinants by rifampicin. ○—○ 3 μg rifampicin per ml; ●—● control

At this time 5×10^4 /ml recombinants were demonstrated in the control culture and only 7×10^1 /ml recombinants in the culture containing rifampicin. In contrast, about 10 times less cells were demonstrated after 3 hr of incubation in the culture growing with rifampicin than in the control culture. Incubation for more than 18 hr was ineffective in increasing the difference in the number of recombinants growing with and without rifampicin.

No curing activity of rifamycin or its derivatives could be demonstrated. There was no loss of resistance in 500 colonies grown from the donor strain after incubation overnight in the presence of rifampicin (3 $\mu\text{g/ml}$) or its derivatives (2 $\mu\text{g/ml}$).

Discussion

The R factor exhibits a number of properties; it displays autonomous replication, synthesises pili, and carries genes determining antibiotic resistance [5-7].

Rifamycin and its derivatives inhibit protein synthesis by acting on DNA-dependent RNA polymerase [8, 9]. JOHNSTON and RICHMOND [10] found that rifampicin at concentrations of about 0.01-0.25 $\mu\text{g/ml}$ effectively eliminated the penicillinase plasmid of staphylococcus. KRČMÉRY and JANOUŠKOVÁ [3] reported that rifampicin prevented the transfer of R factor in *Salmonella typhi-murium* and *E. coli* and eliminated the R factor from the host cells. According to FIETTA *et al.* [11] rifampicin inhibited the adsorption of MS-2 male-specific phage to sex pili. Rifampicin did not affect the phage itself but prevented the formation of sex pili to which the phage had adsorbed. They suggested that the inhibitory effect of rifampicin on the transfer of R and F factors was due to the disturbed pilus formation. It was shown by LÖSER *et al.* [12] that levallorphan also inhibited the adsorption of MS-2 phage and strongly inhibited the transfer of R factor from *Proteus rettgeri* to *E. coli* and this effect was due to the inhibition of mating pair formation.

We have not been able to show any curative activity of rifamycin and its derivatives. These observations were in contrast to those of KRČMÉRY and JANOUŠKOVÁ [3]. The discrepancy may be explained by the difference of the methods. The curative activity may depend on the strain and the size of inoculum. We could not contradict the curative activity of rifampicin, but in our experiments such an effect was not observed.

The inhibitory effect of rifampicin was not as significant in the first few hours of conjugation as it was after 18 hr incubation. At this time the number of the recombinants obtained from cultures with and without rifampicin differed markedly, while the difference was much less after a few hours of incubation. The number of recombinants growing in the presence of rifampicin is about the same after 5 hr and after 18 hr. From these data we have to conclude that rifampicin did not inhibit the conjugation at its onset, and conjugation and formation of the mating pairs were stopped later. We suggest therefore, that at the time of mixing the mating pairs could form with the help of pili already present. As pilus formation or pilus function was disturbed, the formation of mating pairs decreased and this resulted in all inhibition of R factor transfer. The data obtained by KRČMÉRY and JANOUŠKOVÁ [3] confirm

this suggestion. They found that the inhibition of conjugational R factor transfer was more effective when rifampicin was added 5–10 min before the onset of conjugation.

We assume that rifampicin inhibited pilus function or pilus formation and this effect was responsible for the inhibition of R factor transfer. It is likely that rifamycin and rifo acted by the same mechanism.

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